INTERACTIONS AND FUNCTION
OF
NUCLEOTIDE EXCISION REPAIR COMPLEXES

Sofia Jorge de Moura Miguez Araújo

September 1999

Imperial Cancer Research Fund
Clare Hall Laboratories
South Mimms, Hertfordshire
United Kingdom
I would like to thank everyone who helped me during the past four years of my graduate studies.

A special THANK YOU to my Ph.D. supervisor Rick Wood for all the help and advice during the course of this study and being very supportive during the preparation of this thesis (and for having read it in record time!). I would also like to thank members of the laboratory, present and past, for their help and friendship (and the many good moments both in the lab and in the pub!... and for putting up with my singing!): Abdou Aboussekhra, Rafael Ariza, Hiroshi Asahina, Dawn Batty, Maureen Biggerstaff, Liz Evans, Pierre-Henri-Gaillard, Daniela Gunz, Beate Köberle, Isa Kuraoka, Federica Marini, Jonathan Moggs, Jane Sandall, Mahmud Shivji and John Wittschieben.

I would like to thank many people at Clare Hall Laboratories for their help, advice and friendship. As they are too numerous to list, I will mention by name only John Nicholson for the brilliant job done with these photos (and for doing them in record time!) and everybody in Cell Production Services for the endless supply of cells.

I am extremely fortunate to have been a member of the Programa Gulbenkian de Doutoramento em Biologia e Medicina. I thank Dr. António Coutinho and Dr. Alexandre Quintanilha for this great opportunity and for having started this kind of Ph.D. programme in Portugal. A special thanks (e um beijinho!) to Dr.ª Maria José Marinho and Greta Martins. Thank you to all my colleagues from the "PGDBM-Carvalho Guerra" year for the great times we had in Portugal and for all the "group emails".

I would like to give special thanks to my friends in London (and Rui in Brighton - para ti é um obrigadão) who were essential for my happiness and mental health during the last four years (can't wait to go down the pub again!). To my family and friends in Portugal and throughout the world, thank you for making me feel that friendship is stronger than distance! Um beijinho especial para a Ticha, o Jaime e a Joana por estarem sempre tão perto.

Last but not least I thank Richard Hampson for everything...

(and for having read this thesis and for putting up with my stress and for being so supportive and... "é o pau, é a pedra, é o fim do caminho...")

I dedicate this thesis to my mother Celeste and my grandmother Zézé for their endless love and support throughout my life. Sem vocês esta tese não existiria!
Abstract of Thesis

Interactions and function of nucleotide excision repair protein complexes

Nucleotide excision repair (NER) is the main pathway by which mammalian cells remove damage to their DNA caused by UV light and some other mutagens. The initial stages of the NER reaction involve recognition of DNA lesions and cleavage of the damaged DNA strand on either side of the modified base(s) resulting in the release of 24-32-mer oligonucleotides containing the damage (dual incision). The duplex DNA molecule is then restored by semi-conservative DNA synthesis and ligation (repair synthesis).

In order to more fully define which factors are required for dual incision formation I have reconstituted the NER incision reaction using exclusively recombinant proteins on a defined DNA substrate containing a single 1,3-d(GTG) cisplatin adduct. The minimal set of factors necessary for this reaction is RPA, XPA, TFIIH complex (without the CAK kinase subunits), XPC-hHR23B complex, XPG, and ERCC1-XPF complex. TFIIH containing CAK, probably the most physiologically relevant form, can also function in NER. To further define the whole NER reaction, I reconstituted DNA repair synthesis using the recombinant and the highly purified human proteins RPA, RFC, PCNA, DNA ligase I and DNA polymerases δ or ε. Additionally, I purified a factor from HeLa cells, IF7, that significantly stimulates NER incision reactions and found that it is neither necessary for the incision stages of NER nor for repair synthesis.

Xerodermia pigmentosa group D (XP-D) cells, defective in XPD helicase subunit of TFIIH, generally have a relatively high level of UV-induced unscheduled DNA synthesis even though they are deficient in NER. To investigate whether this is due to altered 5′ or 3′ incision activity we studied both XP-D and TTD cells using in vitro NER assays. The majority of XP-D cell extracts tested (bearing different mutations in the XPD gene) were defective in both 3′ and 5′ incisions, though some TTD cell lines made dual incisions weakly. Purified TFIIH containing a defined mutation in the XPD subunit was active in transcription but did not make 3′ or 5′ incisions around a cisplatin lesion.

To investigate the existence of a human repairosome and repair subcomplexes, immunoprecipitation and functional assays were used. In human cells, a functional association consisting of all the above mentioned factors except RPA can be detected at low ionic strength. The strongest interaction found is between TFIIH and XPC.
INDEX

ACKNOWLEDGEMENTS
ABSTRACT OF THESIS
INDEX
LIST OF TABLES & FIGURES
ABBREVIATIONS

CHAPTER I - Introduction
1.1 Nucleotide excision repair (NER) 12
1.2 Proteins involved in the first steps of human NER 16
   1.2.1 XPA 17
   1.2.2 RPA 19
   1.2.3 XPC-hHR23B 22
   1.2.4 TFIIH 26
   1.2.5 XPG 30
   1.2.6 ERCC1-XPF 32
1.3 Mechanism of dual incision formation during NER 34
1.4 Nucleotide excision repair synthesis 37
1.5 The NER/transcription syndromes 38
1.6 Protein complexes in NER 40
   1.6.1 NER complexes in Saccharomyces cerevisiae 40
   1.6.2 Nucleotide excision repair complexes in human cells 44
   1.6.3 Pre-assembled repairosome or sequential assembly of repair factors? 48
   1.6.4 Implications for transcription-coupled repair 50
   1.6.5 NER as a multi-protein machine? 52
1.7 Objectives of this thesis 54

CHAPTER II - Materials and Methods
2.1 Growing and harvesting mammalian cells 56
2.2 Preparation of whole cell extracts 58
2.3 Preparation of nuclear extracts 59
2.4 Preparation of PCNA- and RPA-depleted (CFII) cell extracts 60
2.5 Protein purification 60
   2.5.1 Fractionation of HeLa extracts 60
   2.5.2 Purification of IF7 61
   2.5.3 TFIIH purification 63
2.6 Immunoprecipitation 64
   2.6.1 Protein G beads 64
CHAPTER V - Comparison of the effects in NER of XPD mutations 130
5.1 Introduction 130
5.2 Cell lines with mutant XPD protein and their activity in dual incision 132
5.3 NER activity of TFIIH purified from cells with mutant XPB, XPD and p44 137
5.4 ATP binding site (K48R) XPD mutation 140
5.5 The 3' and 5' uncoupled incision activity of XPD mutants 145
5.6 Repair synthesis activity of XP-D cell extracts 150
5.7 Discussion 153
   5.7.1 XPD mutations in NER 153
   5.7.2 NER activity versus UDS levels 155

CHAPTER VI - Nucleotide excision repair protein complexes 159
6.1 Introduction 159
6.2 TFIIH immunoprecipitation from HeLa cell extracts 161
   6.2.1 Presence of other NER factors 161
   6.2.2 Functional NER interactions 166
6.3 XPG immunoprecipitated from HeLa cell extracts 170
6.4 Strong interaction between TFIIH and XPC-hHR23B 170
6.5 TFIIH immunoprecipitated from lymphoblastoid cells 173
6.6 TFIIH immunoprecipitated from XP-A defective cells 176
6.7 Discussion 179

CHAPTER VII - Concluding remarks 186
7.1 The minimal set of factors involved in nucleotide excision repair in vitro 186
7.2 The role of CAK in nucleotide excision repair 189
7.3 The role of XPD in nucleotide excision repair 190
7.4 NER protein complexes 191
7.5 Implications of NER in human disease 194

REFERENCES 195

APPENDIX 1 - Recombinant ERCC1-XPF purification 218
APPENDIX 2 - Buffer compositions of pure proteins 219
APPENDIX 3 - Immunoblot quantifications 221
APPENDIX 4 - Curriculum vitae 227
LIST OF TABLES & FIGURES

TABLES:

Table 1.1 - Proteins involved in the initial steps of the nucleotide excision repair reaction 17
Table 1.2 - Interactions between S. cerevisiae NER proteins 42
Table 1.3 - Interactions between mammalian NER proteins 47
Table 2.1 - Mammalian cell lines used for the preparation of whole cell extracts 57
Table 2.2 - Antibodies used in Western Blot and immunoprecipitation procedures 67
Table 3.1 - Concentration of the various fractions from the HeLa cell extract fractionation 82
Table 5.1 - Mutations in XPD gene in the XPD and TTD patient cell lines 132
Table 6.1 - Amounts of repair proteins present in the TFIIH bound fraction 162

FIGURES:

Fig. 1.1 - Schematic representation of processing of damaged DNA during the nucleotide excision repair reaction 13
Fig. 1.2 - A model for nucleotide excision repair in mammalian cells 36
Fig. 1.3 - Schematic representation of the protein-protein interactions between the proteins involved in the dual incision formation of NER in S. cerevisiae 41
Fig. 1.4 - Schematic representation of the protein-protein interactions between the proteins involved in the dual incision formation of NER in human cells 45
Fig. 2.1 - Dual incision assay 72
Fig. 2.2 - Titration of the oligonucleotide complementary to the excised fragment 75
Fig. 3.1 - HeLa fractionation scheme 81
Fig. 3.2 - NER proteins present in HeLa fractionation 83
Fig. 3.3 - Repair synthesis is reconstituted by nA, nB and nC 85
Fig. 3.4 - Repair synthesis is reconstituted by RPA, nB, Q5 and H5 and PCNA or exo-free pol I 87
Fig. 3.5 - Purification scheme of human TFIIH 89
Fig. 3.6 - Activity of IF7 purification fractions in repair synthesis reconstituted with nA, nB, Q5 and H5 90
Fig. 3.7 - Purified IF7 from HeLa cells 91
Fig. 3.8 - IF7 activity in a repair synthesis assay reconstituted with purified proteins and exo-free pol I 94
Fig. 3.9 - IF7 activity detected by Southern blotting 96
Fig. 3.10 - IF7 activity detected in dual incision assay 97
Fig. 3.11 - Comparison of TFIIH Hep and TFIIH Hap activities with and without IF7 99
Fig. 3.12 - Comparison of TFIIH Hep and TFIIH Hap activities under different condition buffers

Fig. 4.1 - TFIIH purification scheme

Fig. 4.2 - Purified proteins used in in vitro reconstitution assays

Fig. 4.3 - TFIIH active in transcription is also active in NER

Fig. 4.4 - TFIIH Hap is free of other NER factors

Fig. 4.5 - Recombinant TFIIH is active in NER

Fig. 4.6 - CAK inhibits dual incision formation in the presence of high levels of ATP

Fig. 4.7 - H-8 stimulates dual incision formation in the presence of high levels of ATP and TFIIH Hep

Fig. 4.8 - H-8 stimulates dual incision formation in the presence of high levels of ATP and TFIIH Hap

Fig. 4.9 - TFIIH without the CAK subunit is not inhibited by CAK

Fig. 4.10 - Reconstitution of repair synthesis (I)

Fig. 4.11 - Reconstitution of repair synthesis (II)

Fig. 4.12 - Reconstitution of repair synthesis (III)

Fig. 5.1 - Mutations in the XPD protein

Fig. 5.2 - NER activity of XPD mutant cell lines

Fig. 5.3 - NER activity of immunoprecipitated TFIIH containing mutations in different subunits

Fig. 5.4 - NER activity of purified TFIIH containing defined mutation on the ATP-binding domain

Fig. 5.5 - Scheme representing the sizes of the fragments generated by dual incision or uncoupled incisions on the AvaII cut Pt(GTG) substrate

Fig. 5.6 - Incision activity of XPD mutant cell lines

Fig. 5.7 - Incision activity of purified TFIIH containing mutated XPD helicase

Fig. 5.8 - Repair synthesis activity of XPD mutant cell lines

Fig. 6.1 - TFIIH complex is quantitatively immunoprecipitated from HeLa cell extracts with a cdk7 monoclonal antibody

Fig. 6.2 - Co-immunoprecipitation of TFIIH and other NER factors

Fig. 6.3 - TFIIH interacts with different NER factors in a functional complex

Fig. 6.4 - Adding RPA to the HeLa immunoprecipitates restores their NER activity

Fig. 6.5 - Protein interactions between XPG and other NER factors

Fig. 6.6 - TFIIH interacts with XPC and XPG in a functional complex at 150 mM KCl

Fig. 6.7 - TFIIH interacts strongly with XPC-hHR23B

Fig. 6.8 - Functional interactions of the TFIIH complex in normal lymphoblastoid cells

Fig. 6.9 - Protein-protein interactions in XP-A and XPA complemented XP-A cell extracts

Fig. 6.10 - Schematic representation of protein-protein interactions of human NER factors
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cisplatin</td>
<td>cis-dichlorodiammineplatinum(II)</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CPK</td>
<td>creatine phosphate kinase</td>
</tr>
<tr>
<td>CS</td>
<td>Cockayne syndrome</td>
</tr>
<tr>
<td>dNTP(s)</td>
<td>deoxyribonucleotide triphosphate(s)</td>
</tr>
<tr>
<td>dATP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>2'-deoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>2'-deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>2'-deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethyl aminoethyl</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand break</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERCC</td>
<td>excision repair cross complementing</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FT</td>
<td>flow-through</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast performance liquid chromatography</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutinin</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
</tr>
<tr>
<td>NP 40</td>
<td>Nonidet® P 40 (Triton X-100)</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>Pt(GTG)</td>
<td>1,3-intrastrand d(GpTpG)-cisplatin crosslink</td>
</tr>
<tr>
<td>RPA</td>
<td>replication protein A</td>
</tr>
<tr>
<td>RFC</td>
<td>replication factor C</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TTD</td>
<td>trichothiodystrophy</td>
</tr>
<tr>
<td>UDS</td>
<td>unscheduled DNA synthesis</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WCE</td>
<td>whole cell extracts</td>
</tr>
<tr>
<td>XP</td>
<td>xeroderma pigmentosum</td>
</tr>
</tbody>
</table>
Deoxyribonucleic acid (DNA) is a carrier of genetic information. Led by the need to conserve and transmit information over time, early preconception favoured the idea that this carrier molecule was highly stable. However, DNA is not chemically inert. DNA is a highly reactive molecule that can be damaged by numerous physical and chemical agents, both endogenous and exogenous to the cell. Physical sources of damage include UV radiation that produces cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts and ionising radiation that can result in single- and double-strand breaks. Chemical agents that modify DNA include activated oxygen species generated during oxidative metabolism, alkylating agents and polycyclic aromatic hydrocarbons. Chemical changes induced in the DNA can lead to change in genetic information. Therefore, a variety of mechanisms have evolved in organisms in order to maintain the integrity of their genomes (reviewed in (Lindahl 1993; Friedberg et al. 1995)).

Dedicated DNA repair mechanisms correct DNA damage, maintaining integrity of information and playing a crucial role in survival. These different mechanisms can be divided into three major groups of DNA repair processes: direct reversal of damage, recombinational repair and excision repair.

One example of direct damage reversal is the repair of O\(^6\)-methylguanine. Simple alkylating agents cause alkylation at the O\(^6\) position of guanine, forming a O\(^6\)-methylguanine which will pair ambiguously with both C and T. This methylated base can be removed by O\(^6\)-methylguanine-DNA methyltransferase (MGMT) that removes the methyl group from O\(^6\)-methylguanine in DNA and transfers it to a cysteine residue in the enzyme, in an irreversible reaction that restores normal guanine. Another example
is direct reversal of damage by photoreactivating enzymes which has not been detected in placental mammals. Enzymatic photoreactivation by photolyases can repair UV-induced DNA dimers by splitting the dimer in an electron transfer reaction involving a reduced FAD cofactor. This mechanism represents an extra defense against UV-induced DNA damage.

Recombinational repair acts on double-strand breaks that are caused by ionizing radiation or are chemically induced. Double-strand breaks can be repaired either by homologous recombination (single strand annealing (SSA) or Rad51-mediated strand invasion) or by Ku-dependent non-homologous end-joining (reviewed in (Ivanov and Haber 1997; Nickoloff and Hoekstra 1998)).

Excision repair mechanisms are: base excision repair, mismatch repair and nucleotide excision repair. A common feature between these excision repair mechanisms is that damaged or inappropriate bases on the DNA strand are removed during the repair process and replaced by the normal nucleotide sequence.

In general, base excision repair (BER) removes DNA damage that can arise spontaneously in a cell like base deamination or loss, oxygen free radical attack, or methylation of ring nitrogens (Lindahl 1993). In the BER reaction the damaged base is released by a lesion specific DNA glycosylase, the resulting apurinic or apyrimidinic (AP) site is cleaved by a an AP endonuclease and the molecule restored by DNA repair synthesis and ligation (reviewed in (Lindahl and Wood 1999)).

DNA mismatch repair corrects DNA synthesis errors such as base-base mismatches and short deletion/insertion mutations. Two distinct DNA mismatch correction systems exist in bacteria and eukaryotic cells: short and long patch DNA mismatch repair. In short patch mismatch repair, initiated by specialized glycosylases or endonucleases, the excised tract is 10 nt or less and the reaction has a restricted specificity. Long patch mismatch repair can excise long patches of 10³ bp or more in a reaction initiated by MutS like
proteins and characterised by broad mismatch specificity (Modrich and Lahue 1996).

Nucleotide excision repair (NER) is the major pathway by which mammalian cells remove damage to their DNA caused by UV light and some other mutagens causing "bulky" DNA lesions which mostly distort the DNA helix. The NER reaction involves removal of the damage in the form of an oligonucleotide and hence the name nucleotide excision repair.

1.1 Nucleotide excision repair (NER)

Nucleotide excision repair can act on a variety of DNA lesions. In general, these cause destabilization or distortion of the DNA double helix. UV-light (generally referring to 254 nm UV-C radiation) is the main DNA damaging agent implicated in the formation of lesions repaired by NER. In UV irradiated DNA the two major lesions formed are cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts. The more frequently formed CPDs are removed from the bulk of the genome about 10-fold slower than (6-4) photoproducts (reviewed in (Friedberg et al. 1995; Wood 1996)). This differential repair might be due a (6-4) photoproduct causing more structural distortion than a CPD (Kim and Choi 1995). However, NER does not act only on photoproducts formed by UV light. NER can eliminate other lesions like various cisplatin-purine adducts (Hansson et al. 1991), adducts formed by polycyclic carcinogens such as acetylaminofluorene (AAF) (Hansson et al. 1989; Szymkowski et al. 1993), and adducts of psoralen derivatives (Hansson et al. 1989).

Repair of pyrimidine dimers was first detected in Escherichia coli. Bacterial cells could recover from UV irradiation or had the ability to promote survival of UV-irradiated bacteriophages in a process called host-cell reactivation (reviewed in (Friedberg et al. 1995)). Enzymatic activities responsible for this repair system were identified and found to create
incisions on either side of a damaged base, releasing a 12-13-nucleotide long fragment (Sancar and Rupp 1983). The enzymes involved were designated UvrABC "excinuclease" and the process by which the damaged oligonucleotide was released named "nucleotide excision repair" (NER).

Many features of the process of nucleotide excision repair are known. The initial stages of the NER reaction involve recognition of DNA lesions and cleavage of the damaged DNA strand on either side of the modified base(s) resulting in the release of the oligonucleotide (12-13 nt long in bacteria and 24-32 nt long in mammals) containing the damage. The duplex DNA molecule is then restored by semi-conservative DNA synthesis and ligation (reviewed in (Wood 1996; Wood and Shivji 1997)).

Fig. 1.1 - Schematic representation of processing of damaged DNA during the nucleotide excision repair reaction. A - dual incision step; B - repair synthesis step; i) damage recognition; ii) formation of the open complex; iii) dual incision and excision; the red dot represents any kind of damage acted on by nucleotide excision repair.

The NER reaction can be divided in two major parts conserved in all organisms studied: (A) recognition and incision/excision of damage and (B) repair synthesis (Fig. 1.1). The first part of the NER reaction includes the three steps depicted in figure 1.1 A: i) damage recognition, ii) formation of
an unwound or open DNA structure and iii) placement of the dual incisions around the lesion.

Significant progress has been made in understanding the process by which *E. coli* UvrABC nuclease catalyses the incision of damage from the DNA (reviewed in (Grossman et al. 1998)). The UvrA protein forms a dimer that interacts with UvrB to form a heterotrimeric protein complex, UvrA$_2$B (Orren and Sancar 1989). When UvrA$_2$B binds to the site of damage, conformational changes occur in the protein-DNA complex, leading to the dissociation of UvrA$_2$ and formation of a stable pre-incision UvrB-DNA complex (Oh and Grossman 1986; Orren and Sancar 1990). After release of UvrA, UvrC protein interacts with the UvrB-DNA intermediate and triggers the incision of the damaged DNA strand on both sides of the lesion (Orren and Sancar 1990). The oligonucleotide containing the lesion is then removed from the DNA as a 12-13 bp long fragment and the gap is filled by the dual action of DNA helicase II (UvrD) and DNA polymerase I (Orren et al. 1992).

*Saccharomyces cerevisiae* has been a model organism of choice for the genetic study of eukaryotic NER (Friedberg et al. 1995). In this organism, cells with defects in NER proteins were isolated as UV-sensitive rad mutants. Genes essential for the first steps of NER in this yeast are: RAD1, RAD2, RAD4, RAD10, RAD14, the genes encoding subunits of yeast RPA (RFA1, RFA2 and RFA3) and the genes encoding the subunits of yeast core TFIIH (RAD3, RAD25, SSL1, TFB1, TFB2, TFB3). For homology between human and *S. cerevisiae*, see Table 1.1. In addition, other genes encode NER factors that, although not essential, give rise to moderate UV sensitivity when mutated. These include RAD7, RAD16 and RAD23. Rad7 and Rad16 proteins interact with each other (Guzder et al. 1997; Wang et al. 1997) and are involved in the repair of non-transcribed DNA (Verhage et al. 1994; Wang et al. 1997). It has been proposed that the Rad7-Rad16 complex is
involved in the post-incision events of NER (Reed et al. 1998), but it may also be part of an ATP dependent damage recognition system during NER (Guzder et al. 1998b). Rad4 associates tightly with Rad23 (Guzder et al. 1995b; Wang et al. 1997) which contains a ubiquitin-like region on its N-terminus (Watkins et al. 1993; Schauber et al. 1998). Rad4-Rad23 complex functions in yeast NER where it is required for repair of non-transcribed and transcribed DNA (Mueller and Smerdon 1996; Wang et al. 1997) and has strong affinity for UV-irradiated DNA (Guzder et al. 1998a; Jansen et al. 1998).

NER in *S. cerevisiae* has been reconstituted *in vitro* using purified components and it was shown that a damaged oligonucleotide of about 24-27 nucleotides is released by the combined action of RPA, Rad14, Rad4-Rad23, TFIH, Rad2 and Rad10-Rad1 (Guzder et al. 1995b).

For a long time the study of DNA repair in human cells was limited by the lack of available mutant cell lines defective in repair of DNA damage. In 1968, James Cleaver observed that cultured fibroblasts derived from the skin of a human patient with the disease xeroderma pigmentosum (XP) were defective in excision repair following exposure to UV radiation (Cleaver 1968). This was the first report of a human disease associated with a DNA repair defect. From then on, much attention has been focused on the response of XP cells to DNA damage.

Most of the information that was first obtained on the incision step of mammalian NER derives from studies on cells from patients with XP and also from UV-sensitive Chinese hamster ovary (CHO) cell lines (Friedberg et al. 1995). Fibroblasts from XP patients are UV-sensitive and, with the exception of the class known as XP-V, are deficient in pyrimidine dimer excision and repair synthesis. Like NER deficient *E. coli*, XP cells are also sensitive to a range of chemical agents that produce bulky adducts on DNA (reviewed in (Cleaver and Kraemer 1995)).
To determine if there were different XP complementation groups, nucleotide excision repair was examined in the nuclei of heterokaryons formed by fusing cells from different XP patients. Heterokaryons from certain combinations exhibited complementation and increased repair activity, whereas other combinations remained repair deficient. Combinations that would display normal repair after fusion were classified into different complementation groups. Seven complementation groups have been defined on this basis and designated XP-A to XP-G (de Weerd-Kastelein et al. 1972; de Weerd-Kastelein et al. 1974; Keijzer et al. 1979). The variant complementation group XP-V is not defective in excision repair of UV induced DNA damage (Lehmann et al. 1975), but in a specialised DNA polymerase that bypasses, rather than removes, damage from the DNA (Masutani et al. 1999). Other mutants are the laboratory isolated UV-sensitive rodent cell lines. Human genes that can correct the repair defects of these mutants are called ERCC (excision repair cross-complementing) genes. Some ERCC genes are identical to XP genes and in this case the XP nomenclature is used.

Complementation groups XP-A to XP-G and ERCC1 represent separate proteins that are involved in the initial steps of the NER pathway (Fig. 1.1A) and act together to recognise and remove damaged DNA.

**1.2 Proteins involved in the first steps of human NER**

The molecular basis of the XP complementation groups has been unravelled during recent years and enzymes implicated in this human disease have been isolated. Additionally, other gene products not associated with xeroderma pigmentosum have been shown to take part in the steps leading to the dual incision of DNA damage during the NER reaction. Components of the first steps of human nucleotide excision repair include: XPA, the heterotrimeric RPA, XPC-hHR23B complex, the 6 to 9 subunit
Chapter I - Introduction

TFIIH complex, XPG and ERCC1-XPF nuclease. A high degree of homology exists between the components of the human NER reaction and the 
_\textit{S. cerevisiae}_ repair factors (Table 1.1).

<table>
<thead>
<tr>
<th>NER factor</th>
<th>Activity</th>
<th>components</th>
<th>number of amino acids</th>
<th>S. \textit{cerevisiae} homologue</th>
</tr>
</thead>
<tbody>
<tr>
<td>XPA</td>
<td>affinity for damaged DNA</td>
<td>XPA</td>
<td>273</td>
<td>Rad14</td>
</tr>
<tr>
<td>RPA</td>
<td>ssDNA binding</td>
<td>RPA70</td>
<td>616</td>
<td>Rfa1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RPA32</td>
<td>270</td>
<td>Rfa2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RPA14</td>
<td>121</td>
<td>Rfa3</td>
</tr>
<tr>
<td>XPC-hHR23B</td>
<td>affinity for damaged DNA</td>
<td>XPC</td>
<td>940</td>
<td>Rad4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hHR23B</td>
<td>409</td>
<td>Rad23</td>
</tr>
<tr>
<td>core TFIIH</td>
<td>Pol II basal transcription factor</td>
<td>XPB</td>
<td>782</td>
<td>Rad25/Ssl2</td>
</tr>
<tr>
<td></td>
<td>5'→3' and 3'→5' helicase activity</td>
<td>XPD</td>
<td>760</td>
<td>Rad3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p62</td>
<td>548</td>
<td>Tfb1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p52</td>
<td>462</td>
<td>Tfb2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p44</td>
<td>395</td>
<td>Ssl1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p34</td>
<td>303</td>
<td>Tfb4</td>
</tr>
<tr>
<td>CAK</td>
<td>kinase activity</td>
<td>MAT1</td>
<td>309</td>
<td>Tfb3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cdk7</td>
<td>346</td>
<td>Kin28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyclin H</td>
<td>323</td>
<td>Ccl1</td>
</tr>
<tr>
<td>XPG</td>
<td>structure specific endonuclease</td>
<td>XPG</td>
<td>1186</td>
<td>Rad2</td>
</tr>
<tr>
<td>ERCC1-XPF</td>
<td>structure specific endonuclease</td>
<td>ERCC1</td>
<td>297</td>
<td>Rad10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XPF</td>
<td>905</td>
<td>Rad1</td>
</tr>
</tbody>
</table>

Table 1.1 - Mammalian proteins involved in the initial steps of the nucleotide excision repair reaction and homologies with the yeast _\textit{S. cerevisiae}_.

1.2.1 XPA

Patients belonging to XP complementation group A exhibit severe clinical symptoms with both skin and nervous system disorders. XP-A usually corresponds to the most severe clinical form of xeroderma pigmentosum. XP-A cells in culture show diminished survival and very
low unscheduled DNA synthesis (UDS) after UV irradiation (<5% of normal cells) (Cleaver and Kraemer 1995). The human XPA gene was cloned directly by phenotypic complementation of XP-A cells transfected with total genomic mouse DNA (Tanaka et al. 1990).

XPA is a DNA binding protein with some preference for damaged over undamaged dsDNA (Robins et al. 1991; Jones and Wood 1993; Asahina et al. 1994). Increased binding was observed for DNA that had been UV-irradiated or treated with cisplatin and XPA showed higher affinity for (6-4) photoproducts over CPDs (Jones and Wood 1993). Additionally, XPA protein also binds ssDNA with greater affinity than dsDNA (Eker et al. 1992; Jones and Wood 1993). For these reasons it has been suggested that the degree of XPA binding to DNA lesions is related to the extent of helical distortion and/or helix opening of the damaged region (Jones and Wood 1993). XPA binds DNA through its Zn$^{2+}$-finger DNA binding domain, that is necessary for the protein function (Miura et al. 1991; Miyamoto et al. 1992). This motif is also present on the S. cerevisiae XPA homologue, Rad14 (Guzder et al. 1993). Structural studies have defined the minimal DNA binding domain of XPA as the well conserved region spanning from Met-98 to Phe-219 (Kuraoka et al. 1996; Buchko et al. 1998). This region is rich in helical secondary structure and comprises the zinc binding domain (Buchko et al. 1998; Ikekami et al. 1998).

XPA interacts with several other NER factors as will be described in section 1.6. Particularly important is the XPA-RPA interaction that both increases the affinity of RPA for ssDNA (Lee et al. 1995) and the affinity of XPA for damaged DNA (Li et al. 1995a).

XPA deficient mice are defective in NER and highly susceptible to UV-induced skin carcinogenesis, paralleling the phenotype of humans with XP-A (de Vries et al. 1995; Nakane et al. 1995). Accordingly, a rad14Δ mutation in S. cerevisiae generates very high UV-sensitivity and cells bearing this
mutation are defective in removal of UV induced DNA lesions (Bankmann et al. 1992; Reed et al. 1996).

Recently it was shown that two different cell lines derived from cisplatin chemotherapy sensitive human testicular germ cell tumours have a reduced ability to perform the incision steps of NER. This sensitivity to DNA damaging agents, like the chemotherapeutic drug cisplatin, may be explained by the low levels of XPA protein present in these cells, although the reason for the low XPA levels is unknown (Köberle et al. 1999).

For a long time XPA was the only known damage-recognition factor involved in the NER reaction, due to its affinity for damaged DNA. Recent studies (described at the end of section 1.2.3) from different groups have generated contradictory reports as to the relative importance of XPA and XPC (Sugasawa et al. 1998; Wakasugi and Sancar 1999).

### 1.2.2 RPA

RPA (Replication Protein A) is a core component of multiple DNA transactions: replication, repair and recombination (Wold 1997).

RPA was initially isolated as a factor needed for the in vitro replication of SV40 DNA (Fairman and Stillman 1988; Wold and Kelly 1988; Wold et al. 1989). The requirement for RPA in human NER was first demonstrated by using cell extracts in an in vitro NER assay. Repair synthesis of UV-irradiated plasmid DNA was inhibited in the presence of monoclonal antibodies against RPA (Coverley et al. 1991; Coverley et al. 1992). The involvement of RPA in the incision step of NER was first shown by fractionation of an HeLa cell extract on phosphocellulose yielding two fractions: CFI and CFII. CFI fraction was shown to be necessary for NER to occur and to contain RPA and PCNA (Shivji et al. 1992). The involvement of RPA in both stages of NER was further confirmed by reconstituting this reaction with purified components (Aboussekhra et al. 1995; Mu et al. 1995).
RPA is therefore an essential factor for dual incision of damaged DNA with purified human components (Aboussekhra et al. 1995; Mu et al. 1995). Additionally, RPA complex is required for full opening of the DNA structure formed around a DNA lesion that leads to dual incision formation (Evans et al. 1997b; Mu et al. 1997).

Human RPA is a stable complex of three different subunits: RPA70, RPA34 and RPA14 (reviewed in (Wold 1997)). In *S. cerevisiae*, the homologous three subunits of yeast RPA are encoded by essential genes indicating that all three subunits play a very important role in the cell (Brill and Stillman 1991). No human genetic disorders have yet been found that map to any of the RPA loci, although it is conceivable that mild mutations might be found (Wold 1997).

Heterotrimeric RPA binds preferentially to ssDNA and this binding activity was mainly localised to the 70 kDa subunit (Wold and Kelly 1988; Wold et al. 1989; Brill and Stillman 1991; Kim et al. 1994).

RPA complex binding to ssDNA is dependent on the length of the oligomer; the association constant for a 30-mer is about 60-fold higher than for a 10-mer and the DNA binding site for the heterotrimer is about 30 nt long (Kim et al. 1994). The heterotrimeric RPA complex has two coexistent DNA binding modes: a minimal occluded binding site of 8-10 nucleotides and a more stable binding site of about 30 nucleotides as detected by electron microscopy, gel shift and fluorescence quenching assays (Blackwell and Borowiec 1994; Blackwell et al. 1996). Additionally, RPA70 is predicted to form a stable complex with oligonucleotides as short as 8 nt from its crystal structure (Bochkarev et al. 1997). A current model proposes that initially, heterotrimeric RPA will bind to 8-10 nucleotides via RPA70 and then, via the ssDNA binding domains of RPA32 and RPA14 and the Zn$^{2+}$ finger domain of RPA70, bind to the maximum site of 30 nucleotides long (Bochkareva et al. 1998; Brill and Bastin-Shanower 1998; Bochkarev et al. 1999; Lao et al. 1999). This binding to ssDNA is done with defined polarity.
A strong interaction domain, required for initial ssDNA binding, is positioned at the 5' side of the ssDNA-binding region and a weaker binding domain resides at the 3' side. This predicts a 3'→5' progression from initial binding to a stable 30 nt binding mode that has implications for the mechanism of NER (Bochkarev et al. 1997; de Laat et al. 1998b). Furthermore, it has been observed that RPA complex can bind to dsDNA and display a certain degree of unwinding activity that has been described as a helix-destabilizing activity (Georgaki and Hübscher 1993; Treuner et al. 1996). This destabilizing activity is most probably one of the manifestations of the ssDNA binding capacity of RPA and due to its two DNA binding modes. Heterotrimeric RPA might bind to short ssDNA bubbles in the dsDNA that occur transiently or are the result of DNA damage. Following this initial binding RPA further destabilizes the DNA duplex creating a 30 nt binding site to which it stably binds (Lao et al. 1999).

Human RPA has been reported to bind to cisplatin-, AAF- and UV-damaged DNA and a RPA-XPA complex shows cooperativity in binding to DNA containing AAF lesions (Clugston et al. 1992; He et al. 1995; Burns et al. 1996). In light of these observations, RPA in a complex with XPA has been considered a damage recognition factor in NER (He et al. 1995; Li et al. 1995a). A recent report has detected some degree of RPA binding to the damaged strand of a cisplatin-damaged substrate by photocrosslinking (Schweizer et al. 1999). This binding is not increased by addition of XPA. In contrast, independent studies have proposed that RPA complex binds to the undamaged DNA strand during NER (Evans et al. 1997b) and helps direct the nucleases to cut the damaged strand (de Laat et al. 1998b). Most probably both these observations stem from the ssDNA binding activity of RPA, drawn to the damaged site by the helix-destabilizing character of the DNA lesion (Patrick and Turchi 1998; Patrick and Turchi 1999).

The Zn$^{2+}$ finger motif present in RPA70 is required for DNA replication and mismatch repair but not for NER (Lin et al. 1998). This
might be explained by the fact that the Zn$^{2+}$ finger may only be needed for replication elongation and for the interactions between DNA polymerases and RPA. According to the above mentioned polarity of RPA binding the NER reaction may not need the stronger more stable binding stage. Alternatively, maybe just the participation of the other RPA DNA binding motifs is necessary for the second stage of binding during NER. In the initial stages of NER, where a DNA open structure is formed which is about 30 nt long (see section 1.3), the role of RPA could be one of stabilization/protection of this open structure and of interaction with the other NER factors. Along with XPA, RPA may be involved in primary or secondary lesion recognition and in stabilization of DNA open structure during the NER reaction. In the repair synthesis stage RPA is needed as a DNA polymerase co-factor (Shivji et al. 1995).

RPA34 is phosphorylated in a cell cycle dependent manner at the G$_1$-S transition and when cells are subjected to DNA damage (Din et al. 1990; Carty et al. 1994). After being phosphorylated RPA34 subunit tends to separate from the RPA70 and RPA14 (Treuner et al. 1999). The role of this phosphorylation and its physiological consequences are not yet clear. No specific role for RPA14, except for a structural function in heterotrimeric RPA, has been identified to date (Wold 1997).

As a complex involved in a wide variety of cellular processes, RPA interacts with replication proteins, transcriptional activators and repair proteins (reviewed in (Wold 1997) and section 1.6). The interactions between RPA and other NER factors are further discussed in section 1.6 of this chapter.

### 1.2.3 XPC-hHR23B

Xeroderma pigmentosum group C is one of the most common XP groups. Cells from these patients have low levels of excision repair
(generally 10-20% of normal cells), but higher repair capacity than for instance group A cells (Cleaver and Kraemer 1995). This residual repair activity is associated with transcriptionally active DNA (Venema et al. 1990). Repair of the transcribed strand of expressed genes still takes place in XP-C cells (Venema et al. 1991). This repair activity is directly coupled to transcription and can be inhibited by α-amanitin, a known RNA polymerase II inhibitor (Carreau and Hunting 1992).

The XPC gene was isolated by transfection of XP-C cells with a human cDNA expression library and by testing cells for correction of UV-sensitivity (Legerski and Peterson 1992). XPC polypeptide has a predicted molecular mass of 106 kDa (Masutani et al. 1994), however, when an "XP-C complementing factor" was purified from HeLa cells for its ability to complement XP-C cells it was found to have a native molecular mass of about 160 kDa (Shivji et al. 1994). This paradox was explained by the finding that XPC forms a tight complex with the human homologue of Rad23 (hHR23B) (Masutani et al. 1994). hHR23B is one of the two human homologues of the yeast NER factor Rad23, the other is hHR23A (Masutani et al. 1994). XPC is found in cells mostly complexed with hHR23B, which is much more abundant than XPC protein and, therefore, is also present in the cell in a free form (Masutani et al. 1994; van der Spek et al. 1996). Both human Rad23 homologues can form complexes with XPC and stimulate its repair activity (Sugasawa et al. 1996; Sugasawa et al. 1997). In vivo, however, most XPC complexes with hHR23B, not hHR23A (van der Spek et al. 1996).

Like their S. cerevisiae homologue, both hHR23A and B harbour a ubiquitin-like domain at their amino-termini (Watkins et al. 1993; Masutani et al. 1994). In S. cerevisiae, this moiety is indispensable for the NER activity of Rad23 and mediates the interaction with proteasome subunits, although this domain does not act as a degradation signal (Watkins et al. 1993; Schaubert et al. 1998; Russell et al. 1999). An interaction between the ubiquitin-like domain and the proteasome appears to be required for
optimal NER activity, but this seems to be independent of the proteolytic functions of the proteasome (Russell et al. 1999).

Mice lacking XPC are viable but have high susceptibility to UV-induced carcinogenesis (Sands et al. 1995; Cheo et al. 1997). Knock-outs of either the mouse homologue of hHR23A (Rad23A) or hHR23B (Rad23B) show normal UV-sensitivity, yet mice defective in Rad23B show early post-natal death and males show impaired testicular development. A double knock-out in Rad23A and B is embryonic lethal (Friedberg and Meira 1999).

The role of XPC-hHR23B complex in NER has been a puzzle for a long time. This complex displays high affinity for ssDNA and dsDNA, showing preference for UV-damaged DNA (Shivji et al. 1994; Reardon et al. 1996b). Recently, XPC-hHR23B was shown to preferentially bind UV-, AAF- and cisplatin-damaged DNA over undamaged DNA. The preference of this complex for AAF-damaged DNA was at least 10-fold higher than for the undamaged DNA (Sugasawa et al. 1998). Recent measurements by competition assays show a preferential binding to UV-irradiated DNA of at least 400-fold (D. Batty et al., in preparation). The S. cerevisiae homologue, Rad4-Rad23 complex has also been observed to bind AAF- and UV-damaged DNA (Guzder et al. 1998a; Jansen et al. 1998). Both XPC-hHR23B and Rad4-Rad23 are necessary for the repair of UV lesions in vitro in a system using exclusively purified components (Aboussekhra et al. 1995; Guzder et al. 1995b). However, this complex is dispensable for the in vitro repair of a cholesterol-DNA adduct (Mu et al. 1996) and for the repair of a DNA substrate containing a single-stranded bubble around the lesion (Mu and Sancar 1997). Comparisons with its S. cerevisiae homologue, Rad4, show the least sequence homology (Legerski and Peterson 1992), and in contrast to XP-C cells, Rad4 mutants are totally defective in NER (Couto and Friedberg 1989).

The fact that XP-C patients are defective in global genome repair, hints that XPC-hHR23B might be involved in a step that can be accomplished by
the transcription machinery during transcription coupled repair. Since the stalling of the RNA polymerase II is thought to be the trigger recognition signal for NER during transcription coupled repair (Hanawalt and Mellon 1993), perhaps in repair of non-transcribed regions, XPC complex is necessary for damage recognition.

The mechanism of damage recognition is not completely clear. Recently, two contradictory studies involving the roles of XPC-hHR23B, RPA and XPA have been reported. In one study, it is proposed that XPC-hHR23B is the primary damage recognition factor (Sugasawa et al. 1998) and in the other XPA-RPA are proposed to be the damage sensing factors of NER (Wakasugi and Sancar 1999).

In the former study by Sugasawa et al., two AAF-damaged plasmids of different size were separately pre-incubated, one with purified XPC-hHR23B and the other with all NER factors except XPC-hHR23B. These plasmids were mixed and tested for NER. Initial repair was found mainly on the plasmid pre-incubated with XPC-hHR23B, suggesting that initial binding of XPC complex to lesions is a pre-requisite for NER to occur (Sugasawa et al. 1998). DNase I footprinting experiments also suggested that XPC-hHR23B binds directly to DNA damage and changes the DNA conformation around the lesion (Sugasawa et al. 1998). Based on this study it was suggested that XPC-hHR23B initiates NER by recognising and binding to lesions.

In the latter study by Wakasugi et al. the binding of XPA, RPA and XPC-hHR23B to a DNA duplex containing a (6-4) photoproduct was analysed using mobility-shift assays and DNase I footprinting. Additionally different orders of addition of the three putative "damage recognition" factors were tested. These authors found that pre-incubation of damaged DNA with XPA, RPA or XPA+RPA resulted in faster rates of repair than reactions where DNA had been pre-incubated with XPC complex (Wakasugi and Sancar 1999). Based on these experiments and the fact that under the conditions
used these authors were not able to see any DNase I footprint by XPC-hHR23B on the DNA, it was proposed that RPA-XPA complex is the primary recognition factor in NER.

Previously, another study had detected high specificity and stability of a complex between XPA, RPA, XPC-hHR23B, XPG and DNA (Wakasugi and Sancar 1998). This complex demonstrated much higher specificity for damaged over undamaged DNA in comparison with all other factors alone or in different combinations (Wakasugi and Sancar 1998). Perhaps damage recognition is achieved by a combination of these factors, by a higher order complex and not just by one single recognition protein.

The issue of damage recognition in human cells remains to be clarified. Judging by their characteristics RPA, XPA and XPC-hHR23B may be involved in damage recognition. More studies are needed in order to elucidate if any of these factors acts first or if they act together in the important step of damage recognition during NER.

1.2.4 TFIIH

Xeroderma pigmentosum groups B and D cells carry mutations in two subunits of the TFIIH complex - XPB and XPD. XP group B is a very rare complementation group and cells from these patients have very low levels of NER and are highly sensitive to killing by UV-light (Cleaver and Kraemer 1995). XP group D exhibits considerable clinical heterogeneity. Mutations in the XPD gene, can give rise to genetic syndromes other than xeroderma pigmentosum (XP): Cockayne syndrome (XP-CS) and trichothiodistrophy (TTD) (see section 1.5). Cells from XPD patients generally display a level of UDS which is higher than predicted from their UV survival, since UDS levels correspond to repair synthesis (Johnson and Squires 1992; Cleaver and Kraemer 1995). Both XPB and XPD genes were
cloned indirectly by first cloning the gene correcting the hamster groups ERCC3 and ERCC2, respectively (Weber et al. 1988; Weeda et al. 1990).

TFIIH was first identified as a basal transcription initiation factor for protein coding genes (Moncollin et al. 1986; Conaway and Conaway 1989). TFIIH is a nine-subunit protein complex involved in RNA polymerase II transcription, nucleotide excision repair and probably cell cycle regulation (reviewed in (Moncollin et al. 1998)). According to its composition and enzymatic activities, TFIIH can be divided into two major subcomplexes: core TFIIH (constituted by XPB, XPD, p62, p52, p44 and p34 subunits) and CAK (Cdk-activating kinase that includes Cdk7, Cyclin H and MAT1). All nine subunits of TFIIH complex have been cloned (Fischer et al. 1992; Fisher and Morgan 1994; Humbert et al. 1994; Marinoni et al. 1997b) and recently the entire complex has been expressed in insect cells (Tirode et al. 1999).

The TFIIH complex has multiple enzymatic activities. XPB and XPD display DNA dependent ATPase and helicase activities of opposite polarities (XPB - 3'→5' and XPD - 5'→3') (Conaway and Conaway 1989; Schaeffer et al. 1993; Sung et al. 1993; Schaeffer et al. 1994). The CAK subcomplex is a kinase that is capable of phosphorylating the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II during transcription (Lu et al. 1992; Serizawa et al. 1992). Additionally, this kinase has been reported to phosphorylate the cyclin-dependent kinases Cdc2 and Cdk2, an action required for cell cycle to proceed (Fesquet et al. 1993; Poon et al. 1993; Fisher and Morgan 1994; Roy et al. 1994; Tassan et al. 1994; Serizawa et al. 1995), and other cellular components like p53 and TBP (Okhuma and Roeder 1994; Lu et al. 1997). The additional polypeptides present in core TFIIH (p62, p52, p44 and p34) despite having no identified enzymatic function as yet, are involved in NER and transcription as suggested by inhibition of the TFIIH activity in vitro by monoclonal antibodies against p52, p62, p44 and p34 (Fischer et al. 1992; Humbert et al. 1994; Marinoni et al. 1997b). Moreover, the S. cerevisiae TFIIH subunits (Table 1.1) are essential for cell
viability (Guzder et al. 1994; Wang et al. 1995; Sweder et al. 1996; Feaver et al. 1997). Human p34 and p44 contain Zn$^{2+}$-finger motifs (Humbert et al. 1994) and p44 is involved in the regulation of the helicase activity of XPD by physically interacting with it (Coin et al. 1998b). Some XPD mutations (mostly at the C-terminal domain) interfere with the interactions between XPD and p44, reducing the 5'→3' helicase activity of TFIIH complex (Coin et al. 1998b).

Mice with mutant TFIIH complexes are being studied. Knock-out of the mouse homologue of XPD results in pre-implantation lethality (de Boer et al. 1998b). However, a mouse with single amino acid substitution of XPD that mimics the causative allele in a human TTD patient (TTD1BEL) is viable and shows brittle hair with a low cysteine content, as is characteristic of TTD (de Boer et al. 1998a).

The ATPase/helicase activities of TFIIH have a role in both transcription and nucleotide excision repair. These ATP dependent activities are responsible for melting the DNA double helix (Holstege et al. 1996; Evans et al. 1997b). In transcription, TFIIH was suggested to be responsible for the opening of the DNA duplex at a pre-initiation step in a stage called "open complex formation" (Holstege et al. 1996; Holstege et al. 1997). Binding of TFIIH to the pre-initiation transcription complex leads to the formation of a final single-stranded region of about 10-15 nt at the transcription start site (Holstege et al. 1996; Holstege et al. 1997). In NER, TFIIH is responsible for forming the open-structure of about 30 nt around a DNA lesion, in an ATP dependent manner (Evans et al. 1997a; Evans et al. 1997b). This open-complex provides the single stranded region necessary to allow incision/excision to occur (see below). However, studies with pre-melted DNA substrates have shown that TFIIH is still needed for NER to occur, indicating other role(s) for TFIIH apart from open complex formation (Mu and Sancar 1997).
Several TFIIH subcomplexes have been reported to exist after fractionation of cell extracts. The core complex comprises XPB, p62, p52, p44 and p34 or these 5 subunits plus XPD helicase. CAK can be found as a free heterotrimer and as a component of a 9 subunit TFIIH (Adamczewski et al. 1996; Rossignol et al. 1997). Additionally, CAK can also be found binding to XPD (Drapkin et al. 1996; Reardon et al. 1996a). The CAK subcomplex although important for RNA polymerase II transcription initiation, appears to be dispensable for in vitro NER (Mu et al. 1996; Sung et al. 1996).

Recently, a more detailed study of the various TFIIH subunits was made possible by the production of different TFIIH complexes in the recombinant form (also in this thesis - chapter IV). This provided the tools for the study of purified TFIIH complexes lacking functional XPB or XPD, and the kinase subunits (Tirode et al. 1999). Studying the activities of these TFIIH complexes in transcription in vitro led to the conclusion that maximal activity requires the 9 subunits of TFIIH, although the TFIIH complex lacking the CAK subcomplex is still active in ATP-dependent formation of the open-complex and supports a reduced level of transcription (Rossignol et al. 1997). In addition, comparison of the transcription activities of two 6 subunit TFIIH mutants, one containing a point mutation in XPB (K48R) and another containing a point mutation in XPD (K346R), led to further conclusions about the roles of both helicases in transcription. The XPB helicase is required for transcription, in opening the promoter around the start site whereas the XPD helicase is dispensable for transcription, but is needed for transcription stimulation (Moreland et al. 1999; Tirode et al. 1999; Winkler et al. 1999).
1.2.5 XPG

Complementation group G of xeroderma pigmentosum is one of the most rare and clinically heterogeneous forms of this inherited disease. XPG patients exhibit varying phenotypes, from having a very mild defect in DNA repair to being severely affected, and some cases are also associated with Cockayne’s syndrome (see section 1.5) (Cleaver and Kraemer 1995).

The XPG gene encodes for a 134 kDa protein that is essential for the incision steps of human nucleotide excision repair (O’Donovan and Wood 1993; Scherly et al. 1993). XPG is a structure specific endonuclease which cleaves junctions between duplex and single-stranded DNA when the ssDNA is oriented 3'→5' from the junction (O’Donovan et al. 1994a). Numerous artificial DNA substrates can be cleaved in this fashion by XPG; both XPG and its S. cerevisiae homologue Rad2 can cut single-stranded arms protruding from dsDNA in a 3'→5' orientation (O’Donovan et al. 1994a; Habraken et al. 1995; Evans et al. 1997a). In order to act on bubble substrates, XPG requires a minimal opening of 5 nucleotides and cleaves more substrate if the bubble has a 10 nt unpaired region. There is no substantial difference in the cleavage of bubbles with an unpaired region increasing from 10 nt to 30 nt (Evans et al. 1997a).

XPG is a member of the same family of eukaryotic endonucleases as FEN-1 (DNase IV), which is implicated in the processing of Okazaki fragments during replication. Members of this family cut at junctions of duplex and unpaired DNA with similar polarity (Lieber 1997). As FEN-1, XPG was also shown to interact with PCNA, but unlike the stimulation of FEN-1 by PCNA, no effect was found when PCNA was added to XPG nuclease assays (Wu et al. 1996; Evans et al. 1997a; Gary et al. 1999). XPG contains a helix-loop-helix motif that might be important for both its interaction with DNA and with other NER factors (Park et al. 1997).
Consistent with its cleavage properties, XPG acts in NER by making the incision at the 3' border of the open DNA intermediate (O' Donovan et al. 1994a; Evans et al. 1997a). Interestingly, in the absence of XPG, open-complex formation can be formed but there's no 5' incision upon ERCC1-XPF addition, revealing not only a catalytic but also a structural role for this nuclease (Evans et al. 1997b; Mu et al. 1997). Further evidence for a structural role of XPG during NER has been provided by studies using different nuclease mutants. Mutations in the catalytic site of XPG, that completely abolish the junction cutting and 3' incision activities of XPG, still allowed for full open-complex formation and 5' incision by ERCC1-XPF (Wakasugi et al. 1997; Constantinou et al. 1999). Additionally, a D812A active site mutant that is devoid of incision activity was found to stabilize a pre-incision complex (PIC) containing RPA, XPA, XPC-hHR23B and TFIIH (Mu et al. 1997; Wakasugi et al. 1997). Some other catalytic site mutations, carrying the D77E and E791D amino acid substitutions were unable to cut the bubble substrate on their own but were active in dual incision formation in vitro when added to the other NER factors (Constantinou et al. 1999). These studies indicate that independently of its cleavage activity, XPG has a structural role in protein-protein interactions during NER.

Mice lacking the homologue of human XPG exhibit post natal growth failure and premature death (Harada et al. 1999). This is a more acute phenotype than what is shown by XPA deficient mice, perhaps hinting that XPG has another function in the cell besides NER. Cooper et al. reported that XPG is indeed required for transcription coupled repair of oxidative DNA damage of thymine glycol lesions by base excision repair (Cooper et al. 1997). Subsequently, it was found that missense mutations that inactivate the NER nuclease function of XPG did not affect thymine glycol repair or cause CS. Only mutations that gave rise to severely truncated XPG reduced the rate of thymine glycol repair and were associated with CS (Nouspikel et al. 1997). Recently, the involvement of XPG in thymine glycol excision by base
excision repair was reported (Bessho 1999; Klungland et al. 1999). XPG was shown to stimulate the in vitro activity of the BER enzyme hNth1 DNA glycosylase/AP lyase further hinting at a defect in base excision repair in XP-G patients with Cockayne's syndrome.

1.2.6 ERCC1-XPF

Human ERCC1 does not correct any XP complementation group (van Duin et al. 1989). However, it plays a part in human NER being perhaps an essential gene through its involvement in other cellular processes. Rodent cells with a defect in ERCC1 are hypersensitive to UV irradiation (Wood and Burki 1982). XP group F patients have relatively mild clinical symptoms and XP-F cells display comparatively low levels of UDS (10-20%) (Cleaver and Kraemer 1995). Most interestingly ERCC1 or XPF defective cells are also sensitive to interstrand cross-linking agents suggesting they take part in recombination (Collins 1993). Rad1 and Rad10 have been proposed to be involved in a pathway of recombination between direct repeats, called single-strand annealing (SSA), where they have a role on the removal of non-homologous tails (Fishman-Lobell and Haber 1992; Pâques and Haber 1997). Additionally, analysis of recombination between tandem duplications in mammalian cells in the presence and absence of the ERCC1 gene product led to the suggestion that ERCC1 helps to maintain genome integrity by ensuring the correct processing of intermediates in homologous recombination (Sargent et al. 1997).

Both human ERCC1 and XPF have been isolated and are the human homologues of Rad10 and Rad1, respectively (van Duin et al. 1986; Brookman et al. 1996; Sijbers et al. 1996a). ERCC1 and XPF form a stable complex in vitro and in vivo. The amount of ERCC1 protein is reduced in ERCC4 (XPF) defective cell extracts, suggesting an decreased stability of ERCC1 in the absence of its partner (Biggerstaff et al. 1993; van Vuuren et al.
1993; Sijbers et al. 1996b; Yagi et al. 1997). Interaction between the two partners in this complex is mediated by the C-terminal domain (aa 224-297) of ERCC1 and the C-terminal domain (aa 814-905) of XPF as shown by the study of truncated in vitro translated proteins (de Laat et al. 1998a; McCutchen-Maloney et al. 1999). ERCC1-XPF complex formation is established by a direct interaction between these two binding domains (de Laat et al. 1998a).

ERCC1-XPF complex is a structure specific endonuclease which cleaves junctions between duplex and ssDNA when this ssDNA is oriented 5'→3' from the junction (Sijbers et al. 1996a; de Laat et al. 1998a). Like XPG, both ERCC1-XPF and its S. cerevisiae homologue Rad10-Rad1 incise a variety of different DNA structures like DNA bubbles, stem loops and flaps (Bardwell et al. 1994b; Sijbers et al. 1996a; Bessho et al. 1997; de Laat et al. 1998a). One single-stranded arm protruding in either the 3' or the 5' direction is necessary and sufficient to correctly position ERCC1-XPF incisions at the DNA junction (de Laat et al. 1998a).

In NER, ERCC1-XPF catalyses the incision 5' to the DNA lesion (Sijbers et al. 1996a). Unlike XPG, ERCC1-XPF complex is not needed for full open complex formation and can be added to a pre-assembled pre-incision complex (PIC2, that contains all other factors) to perform the 5' incision (Mu et al. 1996; Mu et al. 1997). Extracts from ERCC1 and XPF-defective cells are defective in the 5' incision of NER, but can still form uncoupled 3' incisions and were shown to open the DNA around a cisplatin lesion (Sijbers et al. 1996a; Evans et al. 1997b). Whether XPF or ERCC1 is responsible for the cleavage activity is still matter of debate. The subunit catalysing the incision has been suggested to be XPF in a study that showed endonuclease activity of full length and of the N-terminal 378 amino acids of XPF (McCutchen-Maloney et al. 1999). These are consistent with a report in S. cerevisiae that showed that Rad1 alone can cleave DNA in a structure specific manner (Habraken et al. 1994). However, both these studies disagree with others that
have shown that Rad1 and Rad10 are unstable and inactive unless part of a complex (Davies et al. 1995).

XPF protein has similarities with members of an RNA-helicase superfamily (SF2) and with some residues of ERCC1, although the relevance of this structural trait to the overall activity of the complex is still matter of study (Sgouros et al. 1999).

Mice in which the ERCC1 gene has been "knocked out" or the encoded protein contains an C-terminal domain truncation are viable but have a very reduced life span (McWhir et al. 1993). ERCC1-mutant cells undergo premature senescence and are sensitive to DNA cross linking agents, a phenotype not found in cells just defective in NER, hinting that they have an additional defect, possibly in recombinational repair (Weeda et al. 1997a).

Several NER protein interactions involving ERCC1-XPF have been reported (see section 1.6). Of these, probably the most relevant are with RPA and XPA that might have implications on the nuclease activity of ERCC1-XPF. Recently, it was reported that the DNA binding orientation of RPA is particularly important for the incision reaction. Bound to the undamaged strand, the 3' oriented side of RPA (the "weak" binding side) binds ERCC1-XPF, whereas the 5'-oriented side (the "strong" binding side) binds XPG. RPA also confers some strand specificity to ERCC1-XPF and XPG by stimulating incisions in the damaged strand (the non-RPA bound strand) and blocking ERCC1-XPF mediated incisions in the undamaged strand (de Laat et al. 1998b).

1.3 Mechanism of dual incision formation during NER

As described in the previous sections, the enzymes involved in the first part of the NER reaction are well known. A current model of how these proteins act together to remove damage from the DNA is represented in figure 1.2. Six protein factors (comprising 15-18 polypeptides) are involved
in the steps leading to the excision of the damaged strand during eukaryotic NER (Table 1.1 and Fig. 1.2). In humans, these factors include the heterotrimeric single-stranded DNA binding protein RPA, XPA protein, XPC-hHR23B complex, XPG and ERCC1-XPF nucleases and the 6 to 9 subunit TFIIH complex. XPE/UV-DDB is dispensable for NER in vitro, but weakly stimulates the repair of UV-irradiated DNA (Aboussekhra et al. 1995).

When an intrastrand helix-distorting lesion occurs on a DNA strand (Fig. 1.2A) it is recognised and repaired by the NER machinery to yield undamaged DNA. The mechanism involved in the initial recognition is currently not elucidated (reviewed in (Wood 1999)) and there are contradictory reports (Sugasawa et al. 1998; Wakasugi and Sancar 1999). XPC-hHR23B complex seems likely to be an important initial recognition factor if the lesion is situated in a non-transcribed DNA strand (Sugasawa et al. 1998) (Fig. 1.2B). If the lesion is in the transcribed strand, initial recognition is believed to involve stalling of the elongating RNA polymerase II (Hanawalt and Mellon 1993; Donahue et al. 1994; Selby et al. 1997) and XPC-hHR23B complex is no longer necessary (Mu and Sancar 1997; Sugasawa et al. 1998). Following recognition, DNA around the site of the lesion is unwound or opened asymmetrically in an ATP dependent manner (Fig. 1.2C) (Evans et al. 1997a). This open complex formation is achieved by TFIIH complex, with its two DNA helicase activities XPB (3'→5' polarity) and XPD (5'→3' polarity) (Evans et al. 1997a; Evans et al. 1997b). This step is also dependent on the presence of XPA and RPA and on ATP hydrolysis (Evans et al. 1997b; Wakasugi et al. 1997; Wakasugi and Sancar 1998; Constantinou et al. 1999). During the repair reaction a single stranded character is conferred on a region of about 24-32 nucleotides, forming the substrate for cleavage by the two structure specific endonucleases XPG (3' incision) and ERCC1-XPF (5' incision) that cut near the junction between the single and the double stranded DNA (Fig. 1.2D) (O’ Donovan et al. 1994a; Matsunaga et al. 1995; Sijbers et al. 1996a; Evans et al. 1997a). Once the incisions have been placed,
an oligonucleotide containing the damage, approximately 24-32 nucleotides long, is released (Moggs et al. 1996).

![Diagram of nucleotide excision repair](image)

**Fig. 1.2** - A model for nucleotide excision repair in mammalian cells. From top to bottom: (A) helix-distorting lesion; (B) recognition of damage; (C) opening of a region around the lesion (open-complex formation); (D) dual incision formation; (E) repair synthesis.

It is currently not completely clear whether the NER reaction is accomplished by a sequential assembly of factors, by subcomplexes of them or even by a completely pre-assembled repairosome (Svejstrup et al. 1995; Guzder et al. 1996). Nevertheless, there are many known interactions between NER factors that give insight into the mechanism of eukaryotic nucleotide excision repair and these will be further discussed in section 1.6.
1.4 Nucleotide excision repair synthesis

The nucleotide excision repair reaction does not end with the excision of the oligonucleotide containing the damage. The DNA molecule is restored by replicative DNA synthesis. The second stage of repair involves proteins implicated in mammalian DNA replication.

The DNA polymerase accessory factor proliferating cell nuclear antigen (PCNA) was shown to be involved in NER by mammalian cell extracts (Nichols and Sancar 1992; Shivji et al. 1992). This need for PCNA suggested that repair synthesis is carried out by polymerase δ or ε. PCNA forms part of a replication holoenzyme with DNA polymerase δ, stimulating it (Podust and Hübscher 1993; Waga and Stillman 1994) and confers high processivity to polymerase ε (Wang 1996). In agreement with this, monoclonal neutralising antibodies against DNA polymerase α do not significantly inhibit DNA repair synthesis carried out by cell extracts (Coverley et al. 1992). Also, it was observed that repair could be inhibited by aphidicolin, further suggesting the involvement of polymerases δ or ε in repair synthesis (reviewed in (Wood and Shivji 1997)). Aphidicolin is a specific inhibitor of polymerases α, δ and ε (Wang 1991). In cell-free assays, pol δ and ε can perform the polymerization step on DNA repair intermediates that have already been incised (Shivji et al. 1995). Repair synthesis on these incised intermediates was also shown to be dependent on RPA, PCNA, RFC (Replication Factor C) and could use DNA ligase I in order to seal the nick (Shivji et al. 1995). The proportion of ligated products after synthesis by polymerase δ on incised NER substrates is stimulated by FEN-1 (DNase IV) because pol δ has strand displacement activity (Shivji et al. 1995). Fully reconstituted DNA repair in vitro, using UV-damaged substrates and purified proteins was accomplished by dual incision factors and a combination of DNA polymerase ε, PCNA, RFC, RPA and DNA ligase I (Aboussekhra et al. 1995). In the cell perhaps either pol δ or ε can perform
repair synthesis on a dual incised DNA, as was shown by genetic studies in the yeast *S. cerevisiae* (Budd and Campbell 1995).

In summary, in the second step of NER, gapped DNA is substrate for replicative synthesis by a polymerase δ or ε holoenzyme with the replication factors RPA, PCNA and RFC. The sugar-phosphate backbone is sealed by a DNA ligase (Aboussekhra *et al.* 1995; Shivji *et al.* 1995; Wood and Shivji 1997) which is probably DNA ligase I (Nocentini 1999) (Fig. 1.2 E).

### 1.5 The NER/transcription syndromes

Several human syndromes exist that show hypersensitivity to DNA-damaging agents. Due to their relevance in view of the results presented in this thesis, I describe three human diseases that display defects in repair of damaged DNA and overlap in their molecular defects - xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothiodystrophy (TTD).

Xeroderma pigmentosum (XP) is a rare autosomal recessive disease with seven complementation groups, from A to G and a variant form called XP-V (see above sections). Patients belonging to complementation groups XP-A to XP-G of this disease have sun sensitivity and are prone to sunlight induced skin cancer. XP has a comparatively high incidence in Japan (1:40000) and a lower incidence in Europe and USA (1:250000) (reviewed in (Cleaver and Kraemer 1995; Friedberg *et al.* 1995)). One of the most readily observed cellular phenotypes of cells derived from XP patients is an increased sensitivity to killing following exposure to a wide variety of DNA-damaging agent such as UV and cisplatin. Not all XP cells are equally sensitive to killing by these agents, revealing molecular difference between these cells. For instance, XP-A cells are the most sensitive XP cells, whereas XP-E cells are the least (Cleaver and Kraemer 1995). This can be attributed directly to the difference in the molecular defect between these XP cells.
Whereas XPA protein is absolutely required for the incision/excision of damaged DNA, the factor responsible for XP-E (UV-DDB) has only a modest stimulatory effect in repair of UV-damaged, naked DNA (Aboussekhra et al. 1995) and has even been reported not to have any effect in complementation studies (Kazantsev et al. 1996; Rapic-Otrin et al. 1998). DDB is probably involved in the repair of UV lesions in a chromatin context (Rapic-Otrin et al. 1998). XP clinical features can sometimes occur in conjunction with features from other clinical disorders like Cockayne syndrome and trichothiodystrophy.

Cockayne syndrome (CS) is an NER related disorder and patients exhibit symptoms including sun-sensitivity as well as developmental and neurologic abnormalities. In contrast with XP, CS patients are not predisposed to skin cancer. This classical form of CS is caused by mutations in the CSA or CSB genes (reviewed in (Friedberg et al. 1995)). Another form of the disease is named XP-CS and some patients of XP-B, XP-D and XP-G complementation groups have both the features of xeroderma pigmentosum and of Cockayne's syndrome (reviewed in (Cleaver and Kraemer 1995; Friedberg et al. 1995)). Cells in CS-A and CS-B groups do not preferentially remove DNA damage from the transcribed strand of active genes, they are specifically defective in transcription-coupled repair (van Gool et al. 1994; Balajee et al. 1997). Characterisation of the molecular defect in cells from the XP-CS group is more puzzling due to their defects in factors involved in NER that lead to CS symptoms (discussed in (Wood 1997)).

Patients suffering from the autosomal recessive disease trichothiodystrophy (TTD) have sulphur-deficient brittle hair, short stature, abnormalities in the skin and nervous system; some patients are also sun sensitive (about 50%), but TTD is not associated with cancer (reviewed in (Cleaver and Kraemer 1995; Friedberg et al. 1995)). Three TTD complementation groups are known so far. A single known patient in complementation group TTD-A defines a form of TTD associated with
defects in a gene encoding a factor that either associates tightly with TFIIH or is involved in some TFIIH complex modification (Stefanini et al. 1993; Vermeulen et al. 1994). The other two complementation groups are defined by defects in the XBP or XPD genes. TTD due to defects in the XPD gene is the most common form of the disease and like in XP-CS the major question was how mutations in one gene can be associated with two different clinical syndromes. Currently, mutations in XPD that give rise to XP have been attributed to the helicase domains and mutations that give rise to TTD to the C-terminal domain of this protein (Taylor et al. 1997).

Most interestingly, both CSB-deficient mice and mice with a mutation in XPD that in humans leads to TTD symptoms exhibit CS and TTD characteristics respectively, but in contrast with the human syndrome these mice show increased susceptibility to skin cancer (van der Horst et al. 1997; de Boer et al. 1998a). Further investigation of these mouse models might start to shed light on these NER/transcription syndromes.

1.6 Protein complexes in NER

1.6.1 NER complexes in Saccharomyces cerevisiae

Interactions between S. cerevisiae NER proteins have been studied using various biochemical methods. In Table 1.2, known interactions between the essential NER proteins are listed.

Rad2, Rad4 and Rad14 interact with the yeast transcription-repair factor TFIIH as shown both by immunoprecipitation and traditional chromatographic methods (Bardwell et al. 1994a; Guzder et al. 1995a). Rad2 interacts directly with the Rad25 (Ssl2) and TFB1 subunits of TFIIH as shown by co-immunoprecipitation of in vitro translated proteins (Bardwell et al. 1994a). TFIIH subunits Rad25 and TFB1 interact with Rad23, to create a Rad4-Rad23-TFIIH association. This interaction was detected by co-
immunoprecipitation of \textit{in vitro} translated proteins (Guzder \textit{et al.} 1995a) and is in accordance with the previous observation that no direct interaction was found between \textit{in vitro} translated TFIIH subunits and Rad4, although TFIIH and Rad4 could be co-immunoprecipitated from yeast cell extracts (Bardwell \textit{et al.} 1994a). Furthermore, Rad14 interacts with TFIIH via Rad23, as seen by co-immunoprecipitation from yeast cell extracts (Guzder \textit{et al.} 1995a), suggesting the existence of a higher order complex including Rad2, TFIIH, Rad4-Rad23 and Rad14 in yeast cells (Bardwell \textit{et al.} 1994a; Guzder \textit{et al.} 1995a).

![Schematic representation of reported protein-protein interactions (summarised in Table 1.2) between the proteins involved in the dual incision formation of NER in \textit{S. cerevisiae}.](image)

The yeast nuclease Rad10-Rad1, partially copurifies with Rad14 as shown by gel filtration and this interaction was further confirmed by immunoprecipitation of these fractions with anti-Rad10 antibodies (Guzder \textit{et al.} 1996). Additionally, there is some degree of interaction between Rad4-Rad23 and Rad7-Rad16 complexes (Wang \textit{et al.} 1997). Most of the above interactions were detected by immunoprecipitation at ionic strengths lower than 100 mM salt concentration (the only exception being the interaction between Rad10-Rad1 and Rad14 that was detected at 300 mM potassium acetate).
<table>
<thead>
<tr>
<th>RPA</th>
<th>TFIH</th>
<th>Rad10-Rad1</th>
<th>Rad2</th>
<th>Rad4-Rad23</th>
<th>Rad14</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CoP, GF (Rodriguez et al. 1998)</td>
</tr>
<tr>
<td>Rad14</td>
<td>CoIP (Guzder et al. 1995); CoP, GF (Svejstrup et al. 1995); CoP, GF (Rodriguez et al. 1998)</td>
<td>GF, IP (Guzder et al. 1996); CoP, GF (Rodriguez et al. 1998)</td>
<td>CoP, GF (Rodriguez et al. 1998)</td>
<td>CoIP, IP (Guzder et al. 1995); CoP, GF (Rodriguez et al. 1998)</td>
<td></td>
</tr>
<tr>
<td>Rad4-Rad23</td>
<td>IP (Bardwell et al. 1994); IP, PD, IP (Guzder et al. 1995); CoP, GF (Svejstrup et al. 1995)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rad2</td>
<td>IP (Bardwell et al. 1994); GF, IP (Habraken et al. 1996); CoP, GF (Svejstrup et al. 1995)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rad10-Rad1</td>
<td>CoP, GF (Svejstrup et al. 1995)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 1.2 - Interactions between S. cerevisiae NER proteins responsible for the dual incision of damage.** Known protein-protein interactions between the S. cerevisiae NER proteins are listed according to the detection method. CoIP - immunoprecipitation from cell extracts; IP - immunoprecipitation from a mixture of pure proteins; PD - "pull-down" method - immunoprecipitation or affinity isolation using excess amounts of one of the proteins; CoP - co-purification by traditional chromatographic methods; GF - co-purification on gel filtration (molecular sizing) columns. When the table entry is a protein complex, interactions are listed between that complex and the other proteins, independently of which of the subunits is involved.
There is also evidence for higher order interactions between NER proteins. Svejstrup et al. (Svejstrup et al. 1995) and Rodriguez et al. (Rodriguez et al. 1998) purified NER proteins from S. cerevisiae cell extracts in a high molecular weight form, designated yeast nucleotide excision repairosome. This form was isolated from yeast whole cell extracts in the absence of DNA and without intentional DNA damage to the cells. In the initial study, His-tagged TFB1 was used as a bait to coimmunopurify core TFIIH (TFIIH containing Rad3, Rad25, SSL1, TFB1, TFB2 and TFB3 subunits (Feaver et al. 1993)) with Radi, Rad2, Rad4, Rad10 and Rad14. Presence in a high molecular weight complex was assessed by gel filtration chromatography at 300 mM salt (Svejstrup et al. 1995). NER factors in this complex were identified both by immunoblotting and by functional complementation of yeast extracts defective in these proteins, showing that these proteins are not only present but also in an active form (Svejstrup et al. 1995). However, repairosome complex alone (without complementing extract) was not active in NER assays. The question therefore remains as to whether it is a functional unit in the cellular NER reaction (Svejstrup et al. 1995). Rodriguez and co-workers isolated complexes, also termed repairosomes, by a slightly different strategy. His-tagged Rad14 was used to co-purify some proportion of the cellular Rad1, Rad10, Rad23, Rfa2 (34 kDa subunit of RPA), Rad7, Rad16 and TFIIH (Rad3 and TFB1) as detected by immunoblotting (Rodriguez et al. 1998). These proteins in a yeast nuclear extract also migrated at a high molecular weight upon gel filtration chromatography in 300 mM potassium acetate. The eluted fraction was also able to cleave Y-shaped DNA substrates in the same way as the structure-specific endonucleases Rad2 and Rad10-Rad1 (Bardwell et al. 1994b; Habraken et al. 1995), consistent with the presence of Rad10-Rad1 and suggesting that Rad2 nuclease is also present in this complex (Rodriguez et al. 1998). This complex could nick UV-irradiated DNA in an ATP dependent manner as it is seen with NER proteins (Rodriguez et al. 1998), suggesting
that this fraction represents a higher order complex active in the first steps of NER.

1.6.2 Nucleotide excision repair complexes in human cells

In humans, interactions between the proteins involved in the first steps of NER (Fig. 1.4) have been studied using various biochemical approaches (Table 1.3).

Human transcription-repair factor TFIIH has been shown to interact with XPA, XPC-hHR23B complex and XPG. The TFIIH-XPA interaction has been detected in both HeLa cell extracts and using purified TFIIH and XPA (Park et al. 1995; Nocentini et al. 1997), whereas the TFIIH-XPC-hHR23B interaction has only been observed by some degree of co-purification of these two complexes (Drapkin et al. 1994; van der Spek et al. 1996). Some XPG also co-purifies with TFIIH through several chromatographic steps (Mu et al. 1995). Iyer et al. immunoprecipitated in vitro translated XPG with XPB, XPD, p62 and p44 subunits of TFIIH (Iyer et al. 1996), indicating an interaction mediated by these subunits of TFIIH. The interaction domains of XPG with the TFIIH subunits included the N-terminal part (for the interaction with XPB and XPD) and a C-terminal region comprising amino acids 747-928 (for interactions with XPB, XPD, p62 and p44) (Iyer et al. 1996).

XPG was also found to immunoprecipitate with in vitro translated RPA (He et al. 1995) and to interact with RPA in the presence of DNA (de Laat et al. 1998b). A ternary interaction was also detected between XPA-RPA and XPG using in vitro translated proteins (He et al. 1995).

The interactions between human NER proteins that have been studied in most detail are between RPA, XPA and ERCC1-XPF complex. XPA interacts with ERCC1-XPF and RPA directly as detected by methods including immunoprecipitation (Li et al. 1994; Park and Sancar 1994; He et al. 1995; Li et al. 1995b; Matsuda et al. 1995; Bessho et al. 1997), yeast two
hybrid systems (Li et al. 1994; Li et al. 1995b; Matsuda et al. 1995) and surface plasmon resonance (Saijo et al. 1996). XPA contacts directly with ERCC1 protein (Li et al. 1994; Bessho et al. 1997). The interaction between XPA and RPA is mediated via two subunits of RPA (both p70 and p34) (Saijo et al. 1996), with the C-terminal domain of p34 mediating the strongest interaction (Walther et al. 1999). Recently, a complex between XPA and XPC-hHR23B on damaged DNA was also reported (Wakasugi and Sancar 1999).

As in S. cerevisiae, co-purification of higher order NER complexes has been reported in human cells (Maldonado et al. 1996; He and Ingles 1997). A fraction containing human RNA polymerase II complex, capable of transcription initiation and elongation in vitro upon addition of TBP and TFIIB, included activities that could complement the NER defect of XP-F, XP-G and XP-C cell extracts (Maldonado et al. 1996). The same proteins further co-eluted with TFIIH, RPA, RFC and polymerase ε, on a gel filtration column performed at 500 mM KCl (Maldonado et al. 1996). This complex was not shown to be functional in NER and it also contained irrelevant factors not known to be involved in the NER reaction such as RAD51, Ku70
and Ku80. The physiological relevance of the partial co-purification of so many proteins is uncertain. In another study, HeLa cell extracts were passed over an XPA affinity column at 100 mM NaCl and the bound proteins eluted with 1 M NaCl. These fractions were analysed by immunoblotting and further purified by immunoprecipitation and gel filtration chromatography done at 70 mM KCl (He and Ingles 1997). The XPA affinity column retained some proportion of RPA, ERCC1, XPG, TFIIH and replication proteins including PCNA, ligase I and RFC (He and Ingles 1997). This fraction was able to perform damage specific DNA synthesis on AAAF-treated DNA substrates in vitro (He and Ingles 1997), suggesting the presence of all NER factors needed for dual incision formation and repair synthesis. However, it was unclear what proportion of total NER proteins was retained on the column. Perhaps only a small percentage exists in the high molecular weight form. Interestingly, essentially the same result can be achieved by chromatography on phosphocellulose. A whole cell extract loaded on phosphocellulose at 100 mM KCl and eluted with 1 M KCl contains all NER proteins except RPA and most of the PCNA (Shivji et al. 1992). This XPA column may partially act as an ion-exchange column in a similar way, though some RPA would be retained on the column, because the vast excess of XPA would encourage the well characterised XPA-RPA interaction. Yet the eluate from the XPA affinity column was shown to contain RPA, XPG, TFIIH, PCNA and RFC upon immunoprecipitation with an XPA antibody at 70 mM KCl and these same factors to co-elute on a gel filtration column performed at the same ionic strength.
<table>
<thead>
<tr>
<th></th>
<th>TFIIH</th>
<th>ERCC1-XPF</th>
<th>XPG</th>
<th>XPC-hHR23B</th>
<th>XPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPA</td>
<td></td>
<td>IP (Bessho et al. 1997); GR (de Laat et al. 1998b)</td>
<td>IP (He et al. 1995); GR (de Laat et al. 1998b)</td>
<td></td>
<td>PD, IP (He et al. 1995), IP (Stigger et al. 1998); 2H, IP, CoIP, PD (Matsuda et al. 1995); IP, SPR (Saijo et al. 1996); 2H, PD (Li et al. 1995a); GR, ELISA (Lee et al. 1995); PD, CoIP, GF (He and Ingles 1997); GR (Wakasugi and Sancar 1999)</td>
</tr>
<tr>
<td>XPA</td>
<td>PD, IP (Park et al. 1995); PD, IP (Nocentini et al. 1997); PD, CoIP, GF (He and Ingles 1997)</td>
<td>IP (Bessho et al. 1997); IP, SPR (Saijo et al. 1996); 2H, IP, PD (Li et al. 1994; Li et al. 1995b); PD (Park and Sancar 1994; Park et al. 1995); PD (He and Ingles 1997)</td>
<td>PD, CoIP (He and Ingles 1997)</td>
<td>GR (Wakasugi and Sancar 1999)</td>
<td>PD (He and Ingles 1997)</td>
</tr>
<tr>
<td>XPC-hHR23B</td>
<td>CoP (Drapkin et al. 1994); CoP (van der Spek et al. 1996)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XPG</td>
<td>IP (Iyer et al. 1996); CoP (Mu et al. 1995)</td>
<td>-</td>
<td>CoIP (Constantinou et al. 1999)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.3 - Interactions between mammalian NER proteins. Known protein-protein interactions between NER proteins are listed according to the detection method. CoIP - immunoprecipitation from cell extracts; IP - immunoprecipitation from a mixture of pure proteins; PD - "pull-down" method - immunoprecipitation or affinity isolation using excess amounts of one of the proteins; CoP - co-purification by traditional chromatographic methods; GF - co-purification on gel filtration (molecular sizing) columns; GR - gel retardation assay; 2H - two-hybrid system; SPR - surface plasmon resonance.

When the table entry is a protein complex, interactions are listed between that complex and the other proteins.
1.6.3 Pre-assembled repairosome or sequential assembly of repair factors?

From research with *S. cerevisiae* two rather different models have emerged for the mechanism of NER: sequential assembly of defined repair factors (Guzder *et al.* 1996) or concerted action of a yeast repairosome (Svejstrup *et al.* 1995).

It has been proposed that *S. cerevisiae* NER factors are organised into specific subassemblies, named nucleotide excision factors (NEF) 1, 2, 3 and 4. NEF1 was defined as Rad14 and Rad1-Rad10 complex (Guzder *et al.* 1996); NEF2 as Rad4-Rad23 complex (Guzder *et al.* 1995b; Guzder *et al.* 1998a); NEF3, as the endonuclease Rad2 bound to TFIIH (Habraken *et al.* 1996) and NEF4 as Rad7-Rad16 complex (Guzder *et al.* 1998b). Based on these data a model was proposed whereby NER is catalysed by the sequential assembly of these factors on the DNA containing damage (Guzder *et al.* 1996). During these studies, use of a pre-assembled NEF1 (in comparison to the addition of Rad1-Rad10 and Rad14 individually) increased the efficiency of incision by about 20% on a UV damaged DNA substrate (Guzder *et al.* 1996). This model relies solely on biochemical data of interactions between the various factors and the sequential action of these particular factors on a damaged DNA substrate has not yet been demonstrated.

In human cells, evidence for stable pre-associations of factors is also incomplete. As discussed above, an active repair complex, containing not only specific NER factors but also replication proteins, could be detected by selecting for affinity to immobilised XPA (He and Ingles 1997). Similarly, an RNA polymerase II complex was found associated with transcription, repair and replication factors (Maldonado *et al.* 1996). Yet an alternative approach yield a contrasting result. Human TFIIH was immunoprecipitated in 100 mM KCl with an antibody directed against the HA-tag on a transfected XPB.
Chapter I - Introduction

After overnight elution with the HA epitope peptide, no other NER factors were found to be associated in significant amounts with the TFIIH. Unexpectedly, the only factor detected at the end of this protocol was SUG1, a proteasome subunit (Weeda et al. 1997b). In yeast, Sug1 and Sug2 were co-immunoprecipitated from a whole cell extract with a Rad23 antibody (Russell et al. 1999) but it is not known whether the Sug-Rad23 interactions are direct.

In another type of experiment designed to observe the movements of NER proteins within living cells, ERCC1-XPF was tagged with green fluorescent protein and its mobility monitored by confocal microscopy and fluorescence recovery after photobleaching. The enzyme was found to move freely around the nucleus with a diffusion constant suggesting no continued association with other factors (Houtsmuller et al. 1999). This argues against the presence of a higher order complex containing ERCC1-XPF within cells since the majority of this complex was found in the free form. However, it does not completely rule out the possibility of the existence of a higher order complex formed by a minority of the repair proteins. In the same study XPA and TFIIH were also reported to diffuse freely in the nucleus. Additionally, a significant fraction of ERCC1-XPF was temporarily immobilised after UV irradiation, suggesting that the observed form is active (Houtsmuller et al. 1999).

Like in yeast, a model for NER that relies on the sequential assembly of repair factors has been proposed (Mu et al. 1997; Wakasugi and Sancar 1998). In this model, higher order complexes (of 4 or 5 NER factors) are conceived to form at the site of a DNA lesion during repair. Intermediates containing different NER factors were sequentially detected on a damaged DNA substrate: pre-incision complex 1, PIC1 (containing RPA, XPA, XPC-hHR23B and TFIIH), PIC2 (PIC1 + XPG) and PIC3 (PIC2 + ERCC1-XPF). The NER reaction was reported to progress from PIC1 to PIC2 and finally PIC3 (Mu et al. 1997). Additionally, formation of a footprint or unwound DNA at a
damaged site was reported to be more efficient in the presence of PIC1 or PIC2, than any of its components alone or in different combinations (Wakasugi and Sancar 1998).

The composition of these sub-complexes associated on DNA does not correspond particularly well with the pre-formed NEF factors proposed in yeast and described above. Further uncertainty has recently arisen: XPA and RPA were reported to bind damaged DNA ahead of XPC-hHR23B and TFIIF (Wakasugi and Sancar 1999), while another study strongly indicated that XPC-hHR23B is the first factor to bind (Sugasawa et al. 1998).

At present, much remains to be clarified. Perhaps the co-purification seen in many cases mostly represents the natural affinity of the factors for one another and not necessarily a pre-association in the cell. Which are the functionally relevant interactions between NER factors? By which mechanism does NER occur? In order to answer these questions, more studies are required in which functional complexes are investigated.

1.6.4 Implications for transcription-coupled repair

Both in \textit{S. cerevisiae} and in human cells, first stage NER proteins (recognition, incision-excision of damage) interact with the transcription machinery (Maldonado \textit{et al.} 1996; Rodriguez \textit{et al.} 1998), the replication machinery (Maldonado \textit{et al.} 1996; Gary \textit{et al.} 1997; He and Ingles 1997) and even with proteins from other DNA repair pathways (Maldonado \textit{et al.} 1996; Bertrand \textit{et al.} 1998; Klungland \textit{et al.} 1999). This is not surprising since dual incision and DNA synthesis are coupled during the NER reaction and can be linked to transcription during transcription-coupled NER.

Transcription coupled nucleotide excision repair is a specialised mode of NER that removes DNA adducts significantly faster from the transcribed strand of genes than from the non-transcribed strand or the bulk of the genome (global genome repair). In mammalian cells, the components of
global genome repair are also required for transcription coupled repair with the exception of XPC-hHR23B, which is not required for the repair of transcriptionally active genes (Venema et al. 1990; Venema et al. 1991).

TFIIH is the common component of transcription and NER (reviewed in (Svejstrup et al. 1996)). Different forms of this complex have been found in both yeast and mammals (Feaver et al. 1993; Adamczewski et al. 1996; Rossignol et al. 1997).

In *S. cerevisiae* it was proposed that an interchange between holo-TFIIH (core TFIIH + Rad25 + TFIIK) and the yeast repairosome occurs during transcription-repair coupling (Svejstrup et al. 1995). Holo-TFIIH is involved in transcription and differs from the TFIIH involved in repair (core TFIIH + Rad25) by the presence of the CTD kinase subunits - TFIIK (Kin28, Ccl1 and Tfb3) (Feaver et al. 1993; Svejstrup et al. 1995). When holo-TFIIH associates with RNA polymerase II it interacts with the rest of the transcription initiation machinery, whereas in the presence of DNA damage core-TFIIH + Rad25 might bind NER proteins and become part of the repairosome. This change in TFIIH subunit composition could be initiated by an elongating RNA polymerase II that stalls at the damaged site, even though TFIIH is not part of the complex involved in transcription elongation (Drapkin et al. 1994; Zawel et al. 1995). Transition from elongating transcription to the repair process might be helped by transcription-repair coupling proteins like Rad26 (the yeast homologue of CSB (van Gool et al. 1994)) (Tijsterman et al. 1997). The dual role of TFIIH would provide cells with the potential of limiting transcription initiation in the presence of DNA damage by changing the majority of the TFIIH complexes to a "repair mode". Nevertheless, it is important to note that both the repairosome complex and holo-TFIIH have been isolated from yeast cells in the absence of any damage induction (Svejstrup et al. 1995), showing that both these complexes can coexist in the cell. Supporting the model where core-TFIIH works in repair and holo-TFIIH in transcription is the finding that Kin28 mutants have
normal repair of non-transcribed DNA strands but impaired transcription-coupled repair (Tijsterman et al. 1998) and the fact that purified holo-TFIIH, but not core-TFIIH can relieve the transcription inhibition due to NER in an \textit{in vitro} system (You et al. 1998).

In humans, one of the repairosome complexes isolated was not found to interact with any of the basal transcription factors (He and Ingles 1997), hinting that also in this system, two different TFIIH complexes might coexist - one for transcription and one for repair. Also, CSB protein was shown to be associated with RNA polymerase II containing complex (van Gool et al. 1997) and to interact with XPG (Iyer et al. 1996), thus being able to provide the bridge between the stalled polymerase and the NER machinery/repairosome.

Alternatively, the interactions found between RNA polymerase II and the NER proteins (Maldonado et al. 1996; Rodriguez et al. 1998) argue in favour of a TFIIH complex that is unique and involved in both transcription and NER. Therefore, one can think of a different model, where all these factors interact and where the repairosome might accompany the elongating RNA polymerase II, acting on the DNA lesions when the polymerase stalls.

1.6.5 NER as a multi-protein machine?

Higher order protein complexes have been reported in a series of different cellular processes. Such complexes have been found from replication to transcription, RNA splicing, nuclear transport and translation (reviewed in (Alberts 1998)). For example, during RNA splicing, the ribonucleoprotein spliceosome machine catalyses the various RNA rearrangements that are necessary to excise an intron from a transcript functioning as a whole protein-RNA complex (reviewed in (Staley and Guthrie 1998)). During replication, there is coupling of the various replication factors to form a coordinate replication machine or replisome
Matters are less clear during the transcription process. Although an RNA polymerase II holoenzyme containing all the transcription factors that are necessary for transcription initiation has been reported (Ossipow et al. 1995; Maldonado et al. 1996), many different holoenzymes and subcomplexes have been isolated (reviewed in (Myer and Young 1998)). For example, some subunits of TFIIH were found to colocalise with coiled bodies in HeLa cells (Jordan et al. 1997). Moreover, large structures described as replication "factories" were found in HeLa nuclei that contained DNA polymerases (Hozak et al. 1993) and transcription "factories" containing RNA polymerases were also detected in discrete nuclear regions (Iborra et al. 1996). Such factories appear to be immobilised in distinct regions of the nucleus and the template DNA is envisaged to slide through them during replication or transcription (Cook 1999). Recently quantitative measurements were made of the proportion of RNA polymerase II and its transcription factors in an HeLa cell (Kimura et al. 1999). Using mild, physiological conditions, Kimura et al. isolated different forms of RNA polymerase II holoenzyme, soluble and insoluble. They concluded that significant amounts of polymerase were attached to the nuclear substructure (more than 50%) but that there were few soluble holoenzymes (about 3%). This study argues against the existence of pre-formed holoenzymes but in favour of the holoenzymes being formed on the DNA and being attached to the underlying nuclear substructure (Cook 1999; Kimura et al. 1999).

It is conceivable that NER would function as one of the above mentioned processes - as a "protein-machine" probably working in discrete locations in mammalian cell nuclei. So called "repair factories" have been proposed in mammalian nuclei although scattered more widely and less concentrated in foci than replication factories (Jackson et al. 1994a; Jackson et al. 1994b; Koehler and Hanawalt 1996). In these studies, damaged DNA was shown to be repaired at particular nuclear sites implying a compartmentalisation of repair. However, one can argue that a process such
as NER, that works only in situations when the DNA is damaged, would be more likely to rely on diffusion of factors through the nucleus and formation of transient complexes at sites of damage only when these are needed for the reaction. A process such as DNA replication or transcription necessarily requires a systematic progression along the DNA, whereas DNA repair may well be more efficient if many factors are free to diffuse around the nucleus, removing lesions wherever they are encountered.

Independent of the existence of a fully pre-assembled repairosome that exists as a whole in the cell, in the absence of DNA or damage induction, it is becoming evident that many interactions do occur and that the NER reaction mechanism relies on complex(es) that work coordinately to excise damage from DNA.

1.7 Objectives of this thesis

The initial aims of this thesis work were to purify and characterise one newly found activity involved in NER - IF7. Additionally, the minimal set of factors involved in NER was to be studied. I started working in Rick Wood's laboratory at the Imperial Cancer Research Fund, Clare Hall Laboratories in October 1995. This was only a few months after the NER reaction had been reconstituted in vitro using a set of purified components. During the course of my PhD work I defined the minimal set of factors involved in NER using a single specific located DNA lesion and recombinant factors when possible. Using this minimal set of factors to perform NER incision reactions I studied different TFIIH complexes. In addition, I used a gentle immunoprecipitation method in combination with a defined functional assay to investigate which protein complexes are formed in significant amounts in human cell extracts.

All the results presented in this thesis are my own original work and have been repeated at least once. Section 1.6 of Chapter I is based in a mini-
review Rick Wood and I wrote for Mutation Research - DNA repair (Araújo and Wood 1999) and Chapters IV and VI are to be published (manuscripts in preparation). Part of Chapter 5 has been published in (Winkler et al. 2000).
This chapter summarises the methods used throughout this thesis. Most sections describe the procedures involved in each method. Section 2.14, however, provides a more detailed description of the methods involved in the analysis of nucleotide excision repair in vitro.

2.1 Growing and harvesting mammalian cells

Mammalian cells were grown by Imperial Cancer Research Fund Cell Production Services according to the following procedures.

Human fibroblast and Chinese hamster ovary cells (Table 2.1) were generally harvested when they reached confluency. Monolayers were washed twice with 100 ml of PBSA. 100 ml 0.25% trypsin/PBSA (Gibco BRL) was added after decanting the PBSA. The trypsin solution was decanted as soon as the cells began to round up. 50 ml medium (Table 2.1) + 15% fetal calf serum (FCS) was added and the flasks were gently agitated until cells detached. Cells were harvested by centrifugation at 200 x g for 5 minutes and the pellet was gently resuspended in 50 ml medium + 15% FCS and repelleted. Finally, cells were resuspended in 50 ml cold PBSA (4°C) and pelleted again. Human lymphoblastoid cells (Table 2.1) were grown to a density of 6 to 8 x 10^5 cells/ml and were harvested by centrifugation. The pellet was resuspended in cold PBSA (4°C) and pelleted again.

Cells were periodically checked for viability by exclusion of nigrosin dye. Only cultures whose viability was greater than 95% were used for the preparation of whole cell extracts. Cell lines were also checked for mycoplasma every month and DNA fingerprinting was performed to confirm the identity of each cell line.
<table>
<thead>
<tr>
<th>Cell line (patient)</th>
<th>Cell type</th>
<th>Mutated Repair Gene</th>
<th>Growth conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa S3</td>
<td>epithelial cervical carcinoma</td>
<td>none</td>
<td>RPMI 1640 medium + 5% FCS</td>
</tr>
<tr>
<td>705ori</td>
<td>human lymphoblastoid</td>
<td>none</td>
<td>RPMI 1640 medium + 20% FCS</td>
</tr>
<tr>
<td>GM2345 (XP2OS)</td>
<td>human lymphoblastoid</td>
<td>XPA</td>
<td>RPMI 1640 medium + 10% FCS</td>
</tr>
<tr>
<td>GM2252A (XP11BE)</td>
<td>human lymphoblastoid</td>
<td>XPB</td>
<td>RPMI 1640 medium + 10% FCS</td>
</tr>
<tr>
<td>GM2485 (XP7BE)</td>
<td>human lymphoblastoid</td>
<td>XPD</td>
<td>RPMI 1640 medium + 10% FCS</td>
</tr>
<tr>
<td>(XP8BR)</td>
<td>human lymphoblastoid</td>
<td>XPD</td>
<td>RPMI 1640 medium + 20% FCS</td>
</tr>
<tr>
<td>(XP11NE)</td>
<td>human lymphoblastoid</td>
<td>XPD</td>
<td>RPMI 1640 medium + 20% FCS</td>
</tr>
<tr>
<td>(XP12BR)</td>
<td>human lymphoblastoid</td>
<td>XPD</td>
<td>RPMI 1640 medium + 20% FCS</td>
</tr>
<tr>
<td>(TTD5BR)</td>
<td>human lymphoblastoid</td>
<td>XPD</td>
<td>RPMI 1640 medium + 20% FCS</td>
</tr>
<tr>
<td>UV5-wt (432.12)</td>
<td>Chinese hamster ovary</td>
<td>XPD</td>
<td>E4 medium + 10% FCS + G418</td>
</tr>
<tr>
<td>UV5-K48R (436.1)</td>
<td>Chinese hamster ovary</td>
<td>XPD</td>
<td>E4 medium + 10% FCS + G418</td>
</tr>
<tr>
<td>XP-t3 (XPCS2BA+tXPB)</td>
<td>human fibroblast SV40-transformed</td>
<td>XPB</td>
<td>RPMI 1640 medium + 10% FCS + G418</td>
</tr>
<tr>
<td>XPG83 (XP125LO)</td>
<td>lymphoblastoid</td>
<td>XPG</td>
<td>RPMI 1640 medium + 10% FCS</td>
</tr>
<tr>
<td>XPG415A (XP2BI)</td>
<td>human fibroblast SV40-transformed</td>
<td>XPG</td>
<td>E4 medium + 10% FCS</td>
</tr>
<tr>
<td>CHO 43-3B</td>
<td>Chinese hamster ovary</td>
<td>ERCC1</td>
<td>E4 medium + 10% FCS</td>
</tr>
</tbody>
</table>

Table 2.1. Mammalian cell lines used for the preparation of whole cell extracts. E4 = (Dulbecco's modification of Eagle's medium); FCS = fetal calf serum. Cell lines with the GM prefix were obtained from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, New Jersey). Cell lines UV5-wt, UV5-K48R and XP-t3 were provided by Dr. J.H.J. Hoeijmakers (Winkler et al. 1998). The cell lines XPG83, XPCS1BA, XP8BR, XP1NE, XP12BR and TTD5BR were established by Epstein-Barr virus immortalisation of lymphocytes from patients (in brackets). The Chinese hamster ovary 43-3B line was derived as described (Wood and Burki 1982). Patient numbers are used to identify the cell line throughout this thesis when no other cell line number is available.
2.2 Preparation of whole cell extracts

Whole cell extracts from lymphoblastoid or fibroblast cells were made according to Manley and co-workers (Manley et al. 1983) with some modifications (Wood et al. 1995). Approximately 10⁹ cells (or multiples thereof) were resuspended in cold PBSA (4°C) and pelleted by centrifugation. The packed cell volume was estimated and the cell pellet resuspended in four packed cell volumes (PCV) of 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 (w/v)). Cells were incubated on ice for 20 minutes and then homogenised by 30 to 40 strokes of a Teflon homogeniser (Jencons Scientific) until ~90% were disrupted (as measured by nigrosin dye exclusion). Four packed cell volumes of sucrose-glycerol buffer (50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 25% sucrose (w/v), 50% glycerol (v/v) (Fluka) and 2 mM DTT) were very slowly added to the homogenate. One packed cell volume of saturated ammonium sulfate solution at 4°C (neutralised to pH 7 with 1 M NaOH) was slowly added, stirring at less than one revolution per second. The stirring was continued for 30 to 50 minutes. The mixture was decanted into ultracentrifuge tubes and spun at 42000 rpm (200000 x g) in a SW55 Beckman ultracentrifuge rotor at 2°C for 3 hours (fixed angled rotors like 45Ti or 60Ti were used for larger volumes of extract). The supernatant was removed and its volume measured. Solid powdered ammonium sulfate was added to a final concentration of 0.33 g/ml. The solution was neutralised by adding 10 μl of 1 M NaOH/g ammonium sulfate. After 30 minutes stirring at 4°C the solution was spun at 11000 rpm (20000 x g) in a Sorvall HB4 centrifuge rotor at 4°C for 60 minutes. The supernatant was discarded and the precipitated protein pellet was transferred into dialysis tubing using a disposable 1 ml syringe (Plastipak). Proteins were dialysed against 2 litres of Dialysis Buffer (25 mM Hepes-KOH pH 7.9, 100 mM KCl, 12 mM MgCl₂, 1 mM EDTA, 17%
glycerol (Fluka) and 2 mM DTT) at 4°C for 12 to 14 hours. After dialysis, the extract was spun in a microfuge at 14000 rpm (16000 x g at 4°C) for 10 minutes to remove insoluble proteins. The supernatant was frozen at -80°C in small aliquots (5-20 µl).

2.3 Preparation of nuclear extracts

Nuclear extracts from lymphoblastoid and fibroblast cells were made according to Masutani and co-workers (Masutani et al. 1994) with some modifications. Cell pellets were resuspended in cold PBSA (4°C) and repelleted by centrifugation. The packed cell volume was estimated before resuspending the cell pellet in four packed cell volumes (PCV) of hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM MgCl₂, 5 mM DTT, 2.5 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 10 µg/ml chymostatin and 5% aprotinin (w/v)). Cells were incubated on ice for 20 minutes before breakage by 30 to 40 strokes of a Teflon homogeniser (Jencons Scientific). The mixture was poured into centrifuge tubes and spun at 3000 rpm (1500 x g) in a Sorvall HB4 centrifuge rotor at 4°C for 10 min. The supernatant was removed and the pellet (containing the nuclei) washed twice with Nuclei Wash Buffer (10 mM potassium phosphate pH 7.5, 2 mM DTT and 2.5 mM PMSF), spinning again at 1500 x g for 10 min at 4°C for harvesting. The pellet was then resuspended in two packed cell volumes of Buffer 1 (20 mM Potassium phosphate pH 7.5, 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 (w/v)) and the salt concentration was gradually adjusted to 0.3 M KCl. After 30 minutes stirring at 4°C the solution was centrifuged at 36000 rpm (100000 x g) in a Beckman 45 Ti (or 60 Ti) ultracentrifuge rotor at 2°C for 60 minutes. The pellet was then discarded and Triton X-100 added to a final concentration of 0.01%. Dialysis was carried out in 2 litres of cold Dialysis Buffer (25 mM Hepes-KOH pH 7.9, 100 mM KCl, 12 mM MgCl₂, 1
mM EDTA, 17% glycerol (v/v) (Fluka) and 2 mM DTT) for approximately 12
hours. After dialysis, insoluble proteins were removed by centrifugation at
16000 x g for 10 min at 4°C and the clarified dialysate loaded onto the
appropriate chromatography column.

2.4 Preparation of PCNA- and RPA-depleted (CFII) cell extracts

CFII cell extracts were prepared as described previously (Shivji et al.
1992). Briefly, whole cell extract (from no less then 10^{10} cells), prepared as
described above, was loaded onto a phosphocellulose (Whatman P11)
column equilibrated in Buffer A (25 mM Hepes-KOH (pH 7.8), 1 mM EDTA,
0.01% NP40 (v/v), 10% glycerol (v/v), 1 mM DTT) containing 0.15 M KCl.
After collection of flow-through (FT) fractions (at 0.15 M KCl, named CFI),
bound protein was eluted with Buffer A containing 1.0 M KCl. The peak
protein fractions from the 1.0 KCl elution (named CFII) were pooled and
dialysed against Dialysis Buffer (25 mM Hepes-KOH (pH 7.9), 1 mM EDTA,
17% glycerol (v/v), 1 mM DTT, 12 mM MgCl2) containing 0.1 M KCl. When
necessary, pressure ultrafiltration through Amicon ultrafiltration cells was
employed to concentrate CFII to at least 5 mg/ml prior to dialysis. After
dialysis, the CFII extract was spun at 3000 x g for 10 min at 4°C to remove
insoluble proteins. The supernatant was frozen at -80°C in small aliquots (5-
20 μl). CFII from HeLa cells were prepared by Mahmud K. K. Shivji.

2.5 Protein purification

2.5.1 Fractionation of HeLa extracts

Step 1 - Fresh cell pellets from a 40 l culture of HeLa cells (approximately 2 x
10^{11} cells) were taken and a nuclear extract was made according to section
2.3. After overnight dialysis against buffer A (25 mM Hepes-KOH pH 7.8, 1
mM EDTA, 0.01% NP40 (v/v), 10% glycerol (v/v), 1 mM DTT) containing 150 mM KCl, the clarified dialysate (480 mg of total protein) was loaded onto a 70 ml (dimensions 15 x 2.5 cm; flow rate 2 ml/min) phosphocellulose column (Whatman P11) pre-equilibrated in buffer A/150 mM KCl. The column was washed with the same buffer A containing 150 mM KCl and proteins eluted with increasing KCl concentrations in a stepwise manner (300 mM, 600 mM and 1 M). All fractions were dialysed against buffer A containing 100 mM KCl.

**Step 2** - Protein eluting from phosphocellulose at 600 mM KCl was loaded onto a 5 ml (7 x 1 cm; 0.5 ml/min) Q-sepharose column pre-equilibrated in buffer A/100mM KCl. The column was washed with the same buffer containing 100 mM KCl and elution performed sequentially with buffer A containing 200 mM and 500 mM KCl.

**Step 3** - The flow-through of Q-sepharose column (100 mM) was made up to 100 mM phosphate and loaded onto a 10 ml hydroxyapatite (15 x 1 cm; 0.5 ml/min) column previously equilibrated in buffer B (25 mM Hepes, pH 7.8, 100 mM KCl, 10% glycerol (v/v), 0.01% NP40 (v/v), 1 mM DTT) containing 100 mM phosphate. After the column was washed, proteins were eluted sequentially with buffer B containing 200 mM, 300 mM and 500 mM phosphate. All fractions were dialysed against buffer A containing 100 mM KCl and stored at -80°C for further use.

### 2.5.2 Purification of IF7

**Step 1** - Nuclear extracts were prepared from approximately 2 x 10^{11} cells according to section 2.3. This nuclear extract was dialysed overnight against buffer A (25 mM Hepes-KOH pH 7.8, 1 mM EDTA, 0.01% NP40 (v/v), 10% glycerol (v/v), 1 mM DTT) containing 150 mM KCl and clarified by centrifugation (3000g, 10 min, 4°C). About 2.5 g of nuclear extract (at a concentration of 3.5 mg/ml) were applied onto a 300 ml (15 x 5 cm; 2
ml/min) phosphocellulose column (Whatman P11) previously equilibrated in buffer A containing 150 mM KCl. After washing extensively with the same buffer, the elution is performed sequentially with buffer A containing 400 mM, 650 mM and 1 M KCl. Protein eluting at 650 mM KCl (255 mg) contained the bulk of IF7 activity and was dialysed against buffer B (25 mM Hepes-KOH pH 7.8, 10% glycerol (v/v), 0.01% NP40 (v/v), 1 mM DTT) containing 100 mM phosphate.

Step 2 - After dialysis, the phosphocellulose 650 mM KCl fraction was loaded onto a 40 ml hydroxyapatite column (8 x 2.5 cm; 1 ml/min) equilibrated in buffer B/100 mM phosphate. The column was washed in the same buffer and proteins eluted in two steps at 300 mM and 500 mM phosphate. The active fraction from this column (300 mM phosphate - 150 mg of protein) was dialysed overnight against buffer A/100 mM KCl.

Step 3 - The hydroxyapatite fraction was applied onto a 2 ml (5 x 1 cm; 0.3 ml/min) ssDNA cellulose column. The column was washed in buffer A/100 mM KCl and the proteins progressively eluted with buffer A containing 300 mM and 600 mM KCl. Most of the IF7 activity was present in the 600 mM KCl fraction that was dialysed against buffer A containing 100 mM KCl.

Step 4 - The active fraction from the ssDNA cellulose column was loaded onto a 0.5 ml (1.5 x 1 cm; 0.08 ml/min) dsDNA cellulose column. This column was washed with buffer A/100 mM KCl and eluted sequentially with buffer A containing 300 mM and 600 mM KCl. IF7 activity was present in the fraction eluted with 300 mM KCl. This fraction was dialysed overnight in buffer A containing 50 mM KCl.

Step 5 - After dialysis, the dsDNA cellulose fraction was applied onto a 1 ml (5 x 0.5 cm; 1 ml/min) FPLC MonoQ column (Pharmacia), previously equilibrated in buffer A/50 mM KCl. Proteins were eluted with a 10 ml linear gradient from 50 mM to 500 mM KCl. Most of the activity was in the flow-through (50 mM KCl) of this column.
**Step 6** - This flow-through fraction was loaded onto a 1 ml (5 x 0.5 cm; 1 ml/min) FPLC MonoS column (Pharmacia) and proteins eluted with a 10 ml linear gradient of buffer A from 50 mM to 500 mM KCl. Active fractions from this column (= 300 mM KCl) were pooled and loaded onto a Superose 6 PC column (30 x 0.32 cm; 0.05 ml/min - SmartSystem - Pharmacia) and gel filtration analysis performed in buffer A containing 300 mM KCl. 50 μl fractions were collected and frozen at -80°C for further analysis.

### 2.5.3 TFIIH purification

Purification of TFIIH was performed based on the scheme developed in Dr. J. M. Egly's laboratory in France (Gerard *et al.* 1991; Marinoni *et al.* 1997a). TFIIH complex presence was followed during the purification procedure by immunoblotting against p62 (antibody 3C9).

**Step 1** - Generally, frozen cell pellets from a 60 l culture of HeLa cells (= 5 x 10^10 cells) were taken, thawed in a water bath at 37°C and a whole cell extract made according to section 2.2. Extract (= 70 ml) was dialysed against buffer C (50 mM Tris-HCl pH 7.9, 5 mM MgCl₂, 17.4% glycerol (v/v), 0.5 mM DTT) containing 100 mM KCl, overnight at 4°C, and clarified by centrifugation the following morning. Approximately 750 mg of cell extract (at a concentration of 5 mg/ml) were applied onto a 400 ml (21 x 5 cm; 1 ml/min) Affi-gel® Heparin (BioRad) column previously equilibrated in buffer C/100 mM KCl. Protein elution was performed sequentially with buffer C containing 0.22, 0.4 and 1.0 M KCl. Fraction 0.4 M KCl contained TFIIH complex and was dialysed against buffer D (50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 8.7% glycerol (v/v), 0.5 mM DTT) containing 70 mM KCl.

**Step 2** - After dialysis, the Heparin 0.4 M KCl fraction (370 ml; 150 mg) was loaded onto a 250 ml (13 x 5 cm; 3 ml/min) DEAE Biogel® A (BioRad) column previously equilibrated in buffer D/70 mM KCl. After washing the
column with buffer D/70 mM KCl, proteins are eluted in three steps at 0.2, 0.35 and 1 M KCl.

**Step 3** - The DEAE 0.2 M KCl fraction (150 ml; 85 mg) which contained TFIIH complex, was made up to 50 mM potassium phosphate, pH 6.3. This fraction was then applied onto a 50 ml (10 x 2.6 cm; 3 ml/min) Hi-Load™ SP Sepharose FPLC column (Pharmacia Biotech), equilibrated in buffer E (50 mM potassium phosphate pH 6.3, 0.1 mM EDTA, 8.7 % glycerol (v/v), 0.5 mM DTT) containing 0.2 M KCl. The column was washed with buffer E containing 0.25 M KCl and proteins eluted with a 200 ml linear gradient from 0.25 M KCl to 0.5 M KCl. 7.5 ml fractions were collected and TFIIH peaked around fractions 19-24 (≈ 0.35 M KCl).

**Step 4** - Fractions from the SP Sepharose column, containing TFIIH were pooled and dialysed against buffer F (50 mM Tris-HCl pH 7.6, 0.1 mM EDTA, 50 mM KCl, 10% glycerol (v/v), 0.5 mM DTT). After dialysis, proteins were loaded onto a 5 ml (3 x 1.5 cm; 1 ml/min) Hi-Trap® Heparin column (Pharmacia Biotech) and proteins eluted with a 20 ml, linear gradient from 0 M to 0.9 M ammonium sulphate (AS). 0.5 ml fractions were collected, from the start of the gradient, and TFIIH complex peaked at fractions 25-30 (≈ 0.6 M AS). Peak fractions were pooled and stored at -80°C for further analysis and use.

All the described purification procedures were performed at 4 °C. Protein concentrations were determined by Coomassie® Plus Protein Assay Reagent (PIERCE).

### 2.6 Immunoprecipitation

#### 2.6.1 Protein G beads

Protein G-Sepharose Fast-Flow beads (Pharmacia) were coupled to 12CA5 monoclonal antibody (2 mg of antibody per 1 ml of wet beads), as
described (Harlow and Lane 1989). Coupled antibodies-beads were incubated with the appropriate protein solution (whole cell extract or purified fraction) overnight at 4°C. The resin was washed three times with 10 volumes of ice cold buffer T (25 mM Tris-HCl pH 7.9, 17% glycerol (v/v), 0.5 mM EDTA, 0.2 mM DTT and 5 mM MgCl₂), containing 0.4 M KCl and 0.1% NP40 (v/v) and two times in buffer T containing 0.1 M KCl and 0.01% NP40 (v/v). Bound material was eluted for 2 h at RT in buffer T containing 0.1 M KCl and 0.01% NP40 (v/v), 0.2 mg/ml insulin, 2 mg/ml synthetic peptide corresponding to the HA epitope (sequence YPYDVPDYA), and 1.0 mg/ml aprotinin.

2.6.2 Magnetic beads

Dynabeads® M-450 (Goat anti-mouse IgG) or M-280 (Sheep anti-rabbit IgG) (DYNAL) were incubated with the specific antibody (approximately 0.3-0.4 µg of antibody per 10⁷ beads) overnight at 4°C. Magnetic isolation was performed on a magnetic particle concentrator (DYNAL MPC®). Cell extracts were incubated with the suitable antibody-magnetic beads (10 µg of extract per 4 x 10⁵ beads) for 2 h at RT and then washed three times in buffer W (25 mM Hepes-KOH pH 7.6, 10% glycerol (v/v) and 0.01% Triton X-100 (v/v)) with the appropriate KCl concentration. Beads were resuspended in buffer W/50 mM KCl and used for SDS-PAGE/Western Blot analysis, or in \textit{in vitro} NER assays.

2.7 Polyacrylamide gel electrophoresis

SDS-PAGE gels, for protein separation, containing 37.5:1 acrylamide:bisacrylamide (Amresco or Severn Biotech) and 1% SDS were prepared according to (Sambrook \textit{et al.} 1989) and run in a Mini-Protean II Cell system (BioRad) or Atto system. Proteins were generally separated on 0.75 mm, SDS-polyacrylamide gels for 1-2 h at 100-200 V in 25 mM Tris, 192
mM glycine, 0.1% SDS. Gels were subsequently stained or transferred onto the appropriate membrane (see section 2.8) for analysis. Separated proteins were detected by Coomassie® staining according to (Sambrook et al. 1989) or silver nitrate staining with Silver Stain Plus (BioRad).

Denaturing polyacrylamide gels, for DNA separation, 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).

Agarose gels, for DNA separation, were prepared using a specific percentage of agarose electrophoresis grade (Gibco BRL) and run in TBE buffer (90 mM Tris-borate and 2 mM EDTA). Ethidium bromide (EtBr) (0.25 µg/ml) was added to the gel and running buffer.

2.8 Western Blot

Proteins were separated on SDS-PAGE according to section 2.7 and then transferred to Immobilon P PVDF (Millipore) or nitrocellulose (Hybond™ Amersham Pharmacia biotech) membrane in 25 mM Tris, 192 mM glycine, 20% methanol as described (Sambrook et al. 1989). Following transfer, the membrane was washed with PBSA or TBS and then blocked with 20% dried milk in PBSA or TBS for 1h. The membrane was washed with PBSA or TBS containing 0.01% Tween-20 and then incubated with the suitable dilution of antibody (see Table 2.2). This was followed by further washing and incubation in the appropriate secondary antibody (anti-mouse or anti-rabbit), diluted (generally 1:25000) in PBSA or TBS containing 0.01% Tween-20. Antibody-protein complexes were detected by chemiluminescence (ECL™- Amersham Pharmacia biotech).
Table 2.2 - Antibodies used in Western Blot and immunopurification procedures. * - polyclonal antibody; dilutions used in Western Blot are in PBS or TBS buffer; all TFIIH antibodies were provided by Dr. J.-M. Egly; MO1.1 antibody was initially provided by Dr. Eric Nigg and later purchased from Novocastra; 12CA5 ascites fluid (BabCo); RPA Ab1 was purchased from Oncogene Research products; αRad23 antibody was provided by Dr. E.C. Friedberg.

2.10 Proteins

His-tagged recombinant XPA protein was produced in *E. coli*, purified to homogeneity as described (Jones and Wood 1993) and had a concentration of 450 μg/ml. Recombinant heterotrimeric RPA was produced in *E. coli*, purified as described (Henricksen *et al.* 1994) and had a concentration of 500 μg/ml. Recombinant ERCC1-XPF complex was produced in *E. coli*,
purified through a phosphocellulose column, Ni\(^{2+}\)-NTA column, and MonoQ (FPLC) columns as described in Appendix 1 (M. Biggerstaff, R. Ariza and R. D. Wood, unpublished) and had a concentration of 20 \(\mu\text{g/ml}\).

Recombinant XPG protein was produced in insect cells, purified to homogeneity as described (O’Donovan et al. 1994b; Evans et al. 1997a) and had a concentration of 45 \(\mu\text{g/ml}\). XPC-hHR23B from HeLa cells was purified as described (Masutani et al. 1994) and had a concentration of 10 \(\mu\text{g/ml}\). Recombinant XPC-hHR23B complex was purified by Dawn P. Batty from insect cells, the purification scheme is based on (Sugasawa et al. 1996) and the final concentration of the complex was 15 \(\mu\text{g/ml}\).

Purified human TFIIH (from HeLa cells) Heparin-5PW (Hep) and hydroxyapatite (Hap) fractions (Marinoni et al. 1997a) and recombinant TFIIH (Tirode et al. 1999) were provided by Dr. J.-M. Egly (Strasbourg, France).

Calf thymus or HeLa purified RFC protein (Podust et al. 1992) and calf thymus or HeLa purified polymerase \(\delta\) (Weiser et al. 1991) were provided by Dr. U. Hübscher (Zurich, Switzerland). Purified human polymerase \(\varepsilon\) was provided by Dr. J. Syväöja (Oulu, Finland) and purified from HeLa cells as described (Syväöja and Linn 1989), except that the glycerol gradient centrifugation was performed in the presence of 20 mM potassium phosphate, pH 7.5. Recombinant ligase I (Mackenney et al. 1997) and DNase IV (Robins et al. 1994) were provided by Dr. T. Lindahl (ICRF, UK). The recombinant PCNA was purified from \textit{E. coli} cells as described (Shivji et al. 1992; Biggerstaff and Wood 1998) and had a final concentration of 600 ng/\(\mu\text{l}\).

Buffer compositions of the proteins used in the dual incision assays throughout this thesis are listed in Appendix 2.
2.11 Synthetic oligonucleotides

Synthetic oligonucleotides were synthesised by the Imperial Cancer Research Fund Oligonucleotide Synthesis Unit using the cyanoethyl phosphoramidite method and an Applied Biosystems model 380B DNA synthesiser.

The 24-mer 5'-TCTTCTTCTGTGCACTCTTCTTCT-3' was used for platination and construction of a closed circular duplex DNA containing a single cisplatin crosslink. The 34-mer, named 5GP, 5'-GGGGGAAGAGTGACAGAAGAAGAGGCCTGGTGp-3' (containing a phosphate group on the 3' end to prevent priming) was used to end label the NER excised DNA products (see section 2.14).

2.12 Platination of synthetic oligonucleotides

The synthesis and purification of 24-mer oligonucleotides containing single intrastrand cisplatin-DNA crosslinks was performed as described in (Yarema et al. 1995; Moggs et al. 1996; Shivji et al. 1998b). The 24-mer containing a unique GTG sequence (see section 2.11) was allowed to react at a concentration of 1 mM with a 3-fold molar excess of cisplatin (3 mM) for 16 h at 37°C in a buffer containing 3 mM NaCl, 0.5 mM Na₂HPO₄ and 0.5 mM NaH₂PO₄. Reactions were stopped by adding NaCl to 500 mM. Platinated and non-modified oligonucleotides were separated in preparative denaturing 20% polyacrylamide gels, identified by UV-shadowing (254 nm), excised, eluted and then purified by gel filtration in Sephadex G-25 (Pharmacia) spun columns.
2.13 Construction of DNA substrates to use in NER \textit{in vitro}

2.13.1 Preparation of UV damaged plasmid DNA substrate

Plasmid mixtures containing one larger UV-irradiated and one smaller unirradiated plasmid were produced as described (Wood et al. 1988; Wood et al. 1995). The 2.9 kb pBluescript KS(+) plasmid (Stratagene) was irradiated with 450 J/m² at a concentration of 50 μg/ml in a petri-dish and subsequently treated with an excess of \textit{E. coli} Nth protein for the removal of pyrimidine hydrates. Purification of closed circular UV-irradiated plasmids is achieved by CsCl/EtBr density gradient centrifugation and by sucrose gradients (as described in (Ausubel et al. 1989; Wood et al. 1995)). The 3.7 kb unirradiated pHM14 (Rydberg et al. 1990) was purified from \textit{E. coli} cells using the Plasmid Maxi kit (Qiagen). Equal amounts of purified damaged (pBS) and undamaged (pHM14) plasmid were combined in a mixture containing 50 μg/ml of each plasmid in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) - named plasmid mix.

2.13.2 Construction of closed circular duplex DNA containing single 1,3- intrastrand d(GpTpG)-cisplatin-DNA crosslink

Covalently closed circular DNA plasmids containing a single 1,3-intrastrand d(GpTpG) cisplatin crosslink Pt(GTG) were produced by priming the plus strand of M13mp18GTGx (Moggs et al. 1996), with the platinated oligonucleotide (section 2.12) as described previously (Moggs et al. 1996) (Shivji et al. 1998b). Control DNA substrates (Con-GTG) were produced by the same method using non-modified oligonucleotides. Ten micrograms of plus strand M13 DNA (1 μg/μl) was annealed with a 3 to 5-fold molar excess of 5'-phosphorylated platinated oligonucleotide or non-platinated as a control. Complementary strand synthesis and ligation reactions were
performed in reaction mixtures (120 μl) containing 10 mM Bis-Tris-Propane-HCl (pH 7.9), 10 mM MgCl₂, 1 mM DTT, 2 mM ATP, 12 μg BSA, 600 μM each of dATP, dCTP, dGTP, and TTP, 30 Units T4 DNA polymerase (New England Biolabs) and 36 Units T4 DNA ligase (New England Biolabs) for 4 h at 37°C.

Closed circular DNA was separated by CsCl/EtBr density gradient centrifugation (as described in (Ausubel et al. 1989)) at 56000 rpm in a 70Ti (Beckman) ultracentrifuge rotor (200000 x g) at 18°C for 48 hours. DNA was subsequently purified by consecutive butanol extraction, centrifugation in a Centricon-30 microconcentrator (Amicon) and a Sephadex G50 (Pharmacia) column or MoBiSpin S200 (MoBiTec). DNA substrates were stored at -80°C in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

2.14 Analysis of NER in vitro

Studying human nucleotide excision repair in vitro in a cell-free system with soluble enzymes has many advantages. It can be used to measure in vitro complementation between different repair defective cell extracts and to study the activity of the various repair proteins. For this reason, the development of the first in vitro assay for the study of NER provided a great leap forward in the study of this repair process (Wood et al. 1988). In 1988, Wood and co-workers developed a soluble system based on human cell extracts that can perform UV-dependent DNA excision repair. Basically, this method monitors the formation of repair patches in exogenously added UV-irradiated DNA. Repair synthesis is measured by incubating damaged circular plasmid DNA with cell free extracts or pure proteins in a reaction mixture that includes radiolabelled deoxynucleoside triphosphates and as an internal control unirradiated plasmid DNA (section 2.14.4 and (Wood et al. 1988)). The plasmid DNA substrate used in this assay is UV-irradiated with 450 J/m², producing on average 10 CPDs and 3 (6-4) photoproducts per molecule (Biggerstaff and Wood 1998).
Fig. 2.1 - Dual incision assay.
On the left hand side is a representation of the whole gel and on the right hand side is the “close up” of the area containing the labelled excised products. Throughout this thesis I have chosen to show the results of dual incision assays in the smaller “close up” version; the molecular size markers used are derived from pBR322 - MspI digested; products from the NER incision reaction migrate as 30-34-mers on this gel because the direct labelling procedure adds 4 residues.
More recently, other, more specific methods have been developed and very importantly, the use of plasmids containing one single lesion placed at a unique site in a DNA duplex could be applied. In this way, NER can be readily and specifically measured by detecting the 24-32 nucleotide products of the dual incision reaction (Moggs et al. 1996; Shivji et al. 1998b). This is a simpler reaction than the full repair process, since it does not involve a eukaryotic DNA polymerase holoenzyme or any DNA synthesis. Moreover, the production of 24-32mers is very specific of NER. As a model lesion, 1,3-intrastrand d(GpTpG)-cisplatin crosslink is used, which has been shown to be a good substrate for the vertebrate NER system (Huang et al. 1994; Moggs et al. 1996). Using this substrate (named Pt(GTG) in this thesis), different in vitro NER assays were developed. The first method to analyse NER using this kind of damaged substrate detects repair products with a labelled complementary oligonucleotide by a Southern blot method (section 2.14.2 and (Moggs et al. 1996; Evans et al. 1997b)).

The second method, more recently developed and more used throughout this thesis, is to 3'-end label the excised oligonucleotide directly with radiolabelled dNTP and a DNA polymerase, using a complementary oligonucleotide with a 5'-overhang that serves as a template (section 2.14.1 and (Shivji et al. 1998b)). This method is hereafter called "dual incision assay" and an example of the results is shown in Figure 2.1. This method allows for a direct and sensitive detection of the damaged strand.

The third method (section 2.14.3) is utilised to detect 3' and/or 5' uncoupled incisions, that is, it allows to look at only the 3' or the 5' incision as they are placed on either side of the lesion. To this end the plasmid DNA substrate containing the 1,3-intrastrand d(GpTpG)-cisplatin crosslink is cut in one of its unique restriction sites 3' to the lesion and labelled on the damaged strand. Using this method, a 3' incision can always be detected (either in the presence or in the absence of 5' incision), and a 5' uncoupled
incision (without the 3' incision) can also be detected (Constantinou et al. 1999).

The fourth method, detects repair synthesis on the specific repair patch after excision of the damaged oligomer. Since the cisplatin lesion is placed on a defined place in the plasmid, restriction enzyme cuts can be done and analysis performed on the fragments that correspond to the area surrounding the lesion (section 2.14.5 and (Shivji et al. 1998a)). Incorporation of radiolabel in these fragments can be correlated with specific NER repair synthesis.

2.14.1 Detection of dual incision by end labelling the excised product - dual incision assay

Reaction mixtures were prepared in a volume of 8.5 μl for the fully defined reconstituted reactions or in 10 μl for cell extract complementation. Repair 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 (v/v), 2.5 μg BSA, 0.025% NP40 (v/v) and 2 mM ATP. Complementation reactions were supplemented with 40 mM phosphocreatine (di-Tris salt) and 0.5 μg creatine phosphokinase (type I, Sigma). Each complementation reaction contained 20 to 50 μg of whole cell extract; each reconstituted reaction 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. 1997a)); any changes to these amounts are specified on the figure legends. Following pre-incubation for 10 min at 30°C, 50 ng Pt(GTG) was added and reactions were continued for 90 min at 30°C (5 min and 30 min respectively for complementation reactions). Reactions were stopped by rapid freezing. 6 ng of an oligo complementary to the excised DNA fragment was added to the reaction mixture. This oligo (see section 2.11) contains 4 extra G residues at the 5' end and was annealed to the excised products by
heating at 95°C and gradually cooling the mixtures to RT. The excision products were radiolabelled with 0.1 units of Sequenase v2.0 polymerase (USB) and 1 μCi [α-32P]dCTP (3000 Ci/mmol), separated on a 14% polyacrylamide denaturing gel and visualised by autoradiography as described (Shivji et al. 1998b).

The amounts of complementary oligo (named 5GP -section 2.11) to add in each reaction were titrated in a dual incision assay using a HeLa WCE to perform the incisions (Fig. 2.2). With excess amounts of complementary oligo added to reactions non-specific bands occur (Fig. 2.2 lanes 2 and 3). These bands are probably the result of self labelling of this oligo by folding into a secondary structure. In fact, when incubated alone, this oligo is also subjected to a certain degree of end labelling (Fig. 2.2 lane 10). To minimise these non-specific effects, 6 ng of 5GP were used in all reactions.

Fig. 2.2 - Titration of the oligonucleotide complementary to the excised fragment.
5GP is a 29-mer complementary to the excised NER fragment with a 4-G overhang on the 5' and phosphate group on the 3' end to prevent priming; lane 1 contains non-purified DNA and 6 ng 5GP; lanes 2-9 contain different amounts of 5GP as indicated and incised DNA was purified before the annealing reaction; the annealing reaction in lanes 2-6 was performed at RT for 15 min and followed by end labelling; the end labelling of reactions in lanes 7-9 was performed after 15 min annealing and freezing-thawing of the sample; lane 10 contains only 12 ng of 5GP that were subjected to end labelling reactions 1-7.
2.14.2 Detection of dual incision by Southern hybridisation

Repair reactions equivalent to 5 times the dual incision assay reactions were set up as described above (section 2.14.1). The incubations were performed as in the previously described assay, plasmid DNA was purified from the reaction mixtures and cleaved with HindIII and XhoI restriction enzymes.

DNA purified from incision reactions was separated in denaturing 12% polyacrylamide gels and transferred by capillary transfer for 90 to 180 minutes from the gel to a nylon membrane (Hybond-N+, Amersham) soaked in 0.9 M Tris-borate, 20 mM EDTA. DNA was fixed to the membrane by treatment with 0.4 M NaOH for 20 min, followed by 5x SSC (0.75 M NaCl, 75 mM trisodium citrate) for 2 min. Fixed membranes were incubated for 16 h at 42°C in hybridisation bottles with 40 ml buffer containing 7% SDS, 10% polyethylene glycol 8000, 250 mM NaCl, 130 mM potassium phosphate buffer (pH 7.0) and 100 pmol 32P-labelled oligonucleotides 5'-GAAGAGTGCACAGAAGAAGAGGCCTGG-3'. Membranes were washed for 10 min in 1x SSC (150 mM NaCl, 15 mM trisodium citrate), 0.1% SDS before exposure to X-ray film with intensifying screens or a phosphorimager screen.

2.14.3 Detection of 3' and/or 5' incisions by 3' labelling of the substrate

Single lesion (Pt(GTG)) plasmid (see section 2.13.2) was cut at an Ava II site 140 base pairs 3' from the lesion and the fragment was radiolabelled at the 3' end using 5000 Units of DNA polymerase I (Klenow fragment) and 65 μCi [α-32P] dTTP (3000 Ci/mmol) per μg of Pt(GTG), and purified by a G-50 Sephadex column or MobiSpin S200 (MoBiTec). Labelling efficiency was checked by TCA precipitation (Sambrook et al. 1989). Repair reactions were performed as in dual incision assay (see section 2.13). DNA was purified and
analysed on a denaturing 6% polyacrylamide gel (Evans et al. 1997a), by autoradiography and phosphorimaging.

2.14.4 DNA repair synthesis reactions - UV damaged DNA

Reactions were carried out in a volume of 30 µl for fully reconstituted reactions (or 50 µl for complementation of defective extracts and fractionated system) containing 40 mM HEPES-KOH pH 7.8, 70 mM KCl, 5 mM MgCl₂, 0.9 mM DTT, 0.4 mM EDTA, 7.5 mM MgCl₂ 3.5% glycerol (v/v), 18 µg BSA, 2 mM ATP, 20 µM of each dGTP, dCTP and TTP, 8 µM of dATP, 22 mM phosphocreatine (di-Tris salt, Sigma), 2.5 µg creatine phosphokinase (type I, Sigma) and 2 µCi [α-32P] dATP (3000 Ci/mm mol) as described in (Wood et al. 1995). Each complementation reaction contained 100 to 250 µg of whole cell extract; each fractionation reaction contained the specified amounts of each fraction; each reconstituted reaction contained 500 ng RPA, 250 ng XPA, 30 ng XPC-hHR23B complex, 100 ng XPG, 50 ng ERCC1-XPF complex and 2 µl of HeLa TFIIH (Hep fr. IV (Marinoni et al. 1997a)); any changes to these amounts are specified on the figure legends. 10 µl plasmid mix containing 250 ng of each plasmid (see section 2.13.1) was added and reactions were incubated for 60 min at 30°C (or 90 min for the fractionated system or 3 h for extract complementation). The synthesis step in reconstituted reactions was achieved by 0.05 Units Klenow exonuclease-free (USB) for 30 min at 30°C. Plasmid DNA was purified from the reaction mixtures, linearized with BamHI and separated by electrophoresis overnight (40 V) on a 1% agarose gel containing 0.25 µg/ml ethidium bromide. Data were analysed by autoradiography and phosphorimaging.
2.14.5 Repair synthesis assay - Single lesion Pt(GTG) DNA

Single lesion Pt(GTG) plasmids were incubated with either cell extracts or purified proteins as described in section 2.14.1 for the formation of dual incisions. All reaction mixtures were supplemented with 40 mM phosphocreatine (di-Tris salt) and 0.5 mg creatine phosphokinase (type I, Sigma). Repair synthesis was performed either simultaneously to the incision step (in the case of fully reconstituted reactions) or following the incision step in two stage reactions (with extracts and CFII fractions). In both cases, synthesis was performed in the presence of 2 μCi [α-32P] dCTP (3000 Ci/mmol), 5 mM dCTP and of 20 mM of each: dATP, dGTP and dTTP. Proteins involved in the synthesis step are specified in each figure. Generally, each synthesis reaction contained: 60 ng recombinant PCNA, 50 ng recombinant RPA, 50 ng recombinant DNA ligase I, 0.8 Units RFC and 0.9 Units of DNA polymerase ε or 0.1 Units of DNA polymerase δ. One RFC Unit is defined as the incorporation of 1 nmol dNMP into acid insoluble material in 60 min at 37°C in an RFC dependent replication reaction containing primed circular DNA, RPA, pol δ and PCNA (Hübscher et al. 1999). DNA was digested in 10-15 μl reactions with 5 Units BstNI (New England Biolabs) at 60°C for 2 h prior to separation by electrophoresis on denaturing 14% polyacrylamide gels and analysis by autoradiography and phosphorimaging.
3.1 Introduction

The NER reaction has been reconstituted *in vitro* using purified proteins (Aboussekhra *et al.* 1995; Guzder *et al.* 1995b; Mu *et al.* 1995). In the study performed by Aboussekhra *et al.* the first steps of the reaction were reconstituted with human purified RPA, TFIIH complex, XPC-hHR23B and ERCC1-XPF, and recombinant XPA and XPG. An additional protein fraction, named IF7, was also found to be necessary for this reaction. During this reconstitution, the incision factors were defined by using, for the second part of the repair reaction (DNA synthesis), a simplified synthesis step using only the exonuclease-free Klenow fragment of *E. coli* DNA polymerase I (exo-free pol I) (Aboussekhra *et al.* 1995). The use of this simplified system permitted the study of repair avoiding the addition of the mammalian DNA replication proteins (RPA, PCNA, RFC and DNA polymerase ε) during the second stage of the reaction. Exo-free pol I performs the gap filling DNA synthesis step in a damage dependent manner and provides a simpler way of studying the incision/excision steps (Calsou and Salles 1994; Aboussekhra *et al.* 1995).

IF7 was originally found as a side fraction from the purification of ERCC1-XPF complex from HeLa cells. The ERCC1-XPF containing fraction originated from a CM-sepharose column was fully active in NER, but when this fraction was further purified over a MonoQ column, two activities separated into different fractions. IF7 was in the flow-through and ERCC1-XPF in the bound fraction of this column (Aboussekhra *et al.* 1995). In the absence of IF7, the fully reconstituted repair reaction (where the synthesis step was performed by RPA, PCNA, RFC, DNA polymerase ε and DNA ligase I) showed little or no repair of damaged DNA. However, when exo-free pol I was used to perform the
synthesis step, reactions devoid of IF7 showed uncontrolled nucleotide incorporation in both damaged and undamaged DNA. This was attributed to the capacity of DNA polymerase I to act at a low number of random nicks in DNA and perform strand displacement synthesis in the plasmid circles (Aboussekhra et al. 1995).

Thus, the original IF7 fraction seemed to contain two activities, one that is needed as a NER factor and another that confers specificity to exo-free pol I in a repair synthesis assay.

There were several possibilities for the identity of IF7. Genetic studies from S. cerevisiae suggested it could be the human counterpart of Rad7 and Rad16 (Aboussekhra et al. 1995). It could also be a factor involved in the coupling of dual incision/excision to repair synthesis or just be involved in the synthesis step.

In this chapter, IF7 purification from HeLa cells and its activity in various in vitro NER assays are described. In the end we were unable to identify the factor or indeed establish reproducible conditions for its assay. Before the chapters describing the core results of my thesis (Chapters IV-VI) the IF7 results are summarised here for the benefit of any future investigations in this area.

3.2 HeLa cell extract fractionation

For all XP (XPA to XPG) proteins there are mutant cell lines available and it is possible to test for their activity by complementation analysis. This approach was used to assess for activity during the isolation of most proteins involved in NER (for examples see (Robins et al. 1991; Shivji et al. 1994)). In these complementation assays, the pure protein (or a fraction from the intermediate steps of the purification procedure) is used to complement the repair defect and reconstitute NER in extracts of the defective cell line. There were no IF7 defective cell lines that would allow for the detection of its activity by in vitro complementation. IF7 activity could only be detected in a fully
reconstituted *in vitro* system or by its effect in a reconstituted reaction where the synthesis step was performed by exo-free pol I. At the beginning of this study, the majority of the purified proteins used in the reconstituted assay were not available in sufficient amounts to test for IF7 activity during a whole purification procedure. For this purpose, a fractionation of HeLa nuclear extracts was performed in order to obtain a reliable system to test for fractions during the attempts to purify IF7.

Nuclear extracts from HeLa cells were prepared and subjected to three chromatographic steps (see section 2.5.1) as represented schematically in Figure 3.1.

![HeLa fractionation scheme](image)

**Fig. 3.1 - HeLa fractionation scheme.** Under the fractions needed for reconstitution are the respective NER proteins detected by immunoblot and predicted from previous fractionations.
Fractions from the first column, phosphocellulose, were named nA, nB, nC and nD. These fractions were mixed in different combinations with ultraviolet (UV)-irradiated and non-irradiated DNA in the presence of [α-32P]dATP and ATP to reconstitute the NER process in vitro (Fig. 3.3 and section 2.14.4). A combination of fractions nA, nB and nC was able to perform repair synthesis in the damaged DNA (Fig. 3.3 lane 1). The damage dependent DNA synthesis observed was dependent on XPA protein and therefore could be confirmed as NER. This XPA dependence was demonstrated by the addition of a monoclonal antibody against XPA that inhibits the NER reaction and by the subsequent recovery upon addition of pure recombinant XPA protein (Fig. 3.3 lanes 3-6).

Former studies indicated that most NER proteins would be present in a fraction eluted from a phosphocellulose column at 0.4 M KCl (Aboussekhra et al. 1995). However, both XPC complex and ERCC1-XPF activities are mostly present in a fraction that elutes from a phosphocellulose column at 0.6 M KCl (Shivji et al. 1994; Aboussekhra et al. 1995). Since IF7 was first isolated as a side fraction from the purification of ERCC1-XPF, fraction nC (that elutes at 0.6 M KCl from the phosphocellulose column) was chosen for further fractionation.

Fraction nC was fractionated on a Q-sepharose column to yield Q1, Q2 and Q5, eluted at 0.1 M, 0.2 M and 0.5 M KCl, respectively. The flow-through of this column (fraction Q1) was chosen for fractionation on the next column, because, in the initial study, IF7 was found in the flowthrough fraction of a MonoQ column (Aboussekhra et al. 1995). In a final step of this procedure fraction Q1 was further purified on a hydroxyapatite column yielding fractions H1, H2, H3 and H5 (Fig. 3.1).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>nE</th>
<th>nA</th>
<th>nB</th>
<th>nC</th>
<th>nD</th>
<th>Q1</th>
<th>Q2</th>
<th>Q5</th>
<th>H1</th>
<th>H2</th>
<th>H3</th>
<th>H5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mg/ml)</td>
<td>3.0</td>
<td>4.0</td>
<td>13.0</td>
<td>2.0</td>
<td>1.2</td>
<td>3.0</td>
<td>3.5</td>
<td>7.0</td>
<td>1.6</td>
<td>2.7</td>
<td>3.3</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Table 3.1 - Concentration of the various fractions from the HeLa cell extract fractionation.
Fig. 3.2 - NER proteins present in HeLa fractionation. From top to bottom: blots probed with anti-RPA (p34A), anti-PCNA (PC10), anti-ERCC1 (RW017), anti-p62 (3C9) and anti-XPG (8H7) antibodies; each lane contained 25 μg of each fraction; P = 1 μg purified protein.
All protein fractions from this procedure were assayed for the presence of NER proteins by immunoblotting (Fig. 3.2) and their activities tested by reconstitution of repair synthesis in vitro. By immunoblot analysis and also based on previous studies of the fractionation of human cell extracts, an estimate of which proteins were being isolated in each step of the fractionation procedure could be done (Figs. 3.1 and 3.2). As expected, RPA and PCNA were present in fraction nA (Figs. 3.2 and 3.6). Previous studies (Wold and Kelly 1988; Shivji et al. 1992) had shown that when a HeLa cell extract is fractionated on a phosphocellulose column, RPA and PCNA do not bind and can be isolated in the flowthrough fraction. The same was observed here, both RPA and PCNA were detected in fraction nA (Fig. 3.2), the 0.15 M KCl flow-through of the phosphocellulose column. Accordingly, the activity in nA could be substituted by RPA and PCNA in a repair synthesis assay (Figs. 3.4 and 3.6), further confirming the presence of these proteins in this fraction. Although the p34 subunit of RPA is also detected in fraction Q5, this is not significant in terms of activity as can be observed by the lack of repair synthesis when RPA is omitted from the reactions shown in Figure 3.6 (lane 4).

A bulk of repair factors was detected in fraction nB, namely XPG, ERCC1 and XPA, as observed during previous fractionations of similar kind (Aboussekhra et al. 1995). Detection was performed for ERCC1 only, but since it invariably co-purifies with its partner XPF (Biggerstaff et al. 1993; Sijbers et al. 1996a), the extrapolation was made to ERCC1-XPF. Fraction nC, contained ERCC1-XPF and TFIH complex as detected in fractions from subsequent purification steps (Fig. 3.2). ERCC1-XPF was detected in fraction Q5, meaning that ERCC1-XPF complex eluted from this phosphocellulose at 0.6 M KCl (fraction nC). Also, there was some ERCC1-XPF complex present in fraction nB, showing that this complex was spread over fractions nB and nC.
**Fig. 3.3 - Repair synthesis is reconstituted by nA, nB and nC.** Top panel: EtBr stained gel; bottom panel: autoradiograph; "-" unirradiated plasmid; "+" UV-irradiated plasmid substrate. UV-irradiated and non-irradiated plasmids were incubated with fractionated HeLa cell extracts as described (section 2.14.4); reactions contain 12 µg nA, 40 µg nB and 8 µg nC. Amounts of XPA monoclonal antibody (12F5 - 1 mg/ml) were added as indicated; 100 ng of XPA protein were added where indicated.
TFIIH complex appeared in fractions Q5 and H5 (and a small amount detected in both fractions H2 and H3) (Fig. 3.2) meaning this complex is mainly eluting from phosphocellulose at 0.6 M KCl, consistent with the purification properties of this multisubunit complex (Moncollin et al. 1986; Flores et al. 1992). TFIIH also spread between fractions Q5 and Q1 which is most probably due to part of this complex not binding to Q-sepharose. Neither XPA nor XPC-hHR23B were detected by immunoblot, because good antibodies for blotting were not available at the time. However, from previous studies it was known that XPA activity mainly elutes from a phosphocellulose column at 0.4 M KCl (Robins et al. 1991) and XPC-hHR23B from the same column at 0.6 M KCl (Shivji et al. 1994). For this reason XPA was predicted to be present mainly in fraction nB and XPC-hHR23B in fraction nC.

A fully reconstituted repair reaction was obtained by using nA, nB, Q5 and H5 fractions. Alternatively nA could be replaced by RPA and PCNA (Fig 3.4 lane 1). Being able to substitute fraction nA for RPA and PCNA made it possible to use exo-free pol I for the DNA synthesis step also in this system, because of the PCNA dependence of both polymerase δ and ε (Shivji et al. 1995). Reactions can be reconstituted in two different ways. By using RPA, PCNA, nB, Q5 and H5 repair synthesis is accomplished by the cell’s replication machinery in a PCNA dependent manner (Fig. 3.4 lanes 1-3). In turn, incision reactions can be performed by RPA, nB, Q5 and H5 and the synthesis step achieved by exo-free pol I (Fig. 3.4 lanes 4-8).

In this fractionated system, addition of IF7 is stimulatory, but not absolutely required. In the presence of RPA, PCNA, nB, Q5 and H5 there is damage dependent synthesis in a UV-damaged plasmid (Fig. 3.4 lane 1). However, when IF7 was added to these reactions stimulation was detected (Fig. 3.4 lane 3). The same was observed when synthesis was performed by exo-free pol I. Different IF7 fractions (CF or p2) stimulated the repair signal about 1.5-fold (Fig. 3.4 lanes 4, 6 and 8).
Fig. 3.4 - Repair synthesis is reconstituted by RPA, nB, Q5 and H5 and PCNA or exo-free pol I. UV-irradiated and non-irradiated plasmids were incubated with fractionated HeLa cell extracts as described (section 2.14.4); reactions contain 500 ng RPA, 40 µg nB, 21 µg Q5, 3.5 µg H5, 3 µl IF7 and 60 ng PCNA or 0.05 U of exo-free pol I as indicated; IF7CF and p2 are different preparations of IF7; results were quantified using NIH image software.
IF7 fraction 2 (p2) is a previous IF7 preparation, from a partially purified side fraction of ERCC1-XPF purification (A. Aboussekhra, unpublished results). IF7 CF was an IF7 fraction further purified from the present HeLa cell fractionation (from a fractions H2 and H3 - see below).

Fractions H2 and H3 were shown to substitute for the stimulatory effect of IF7 and, therefore, these were used as starting material for a subsequent purification of IF7 (Fig. 3.6 lane 5). The stimulatory effect of H2+H3 is seen by comparing lanes 2 and 5 (Fig. 3.6). These were pooled and further purified on a phosphocellulose and a ssDNA cellulose column to yield IF7CF (A. Aboussekhra, unpublished results).

Fractions nA, nB, Q5 and H5 were a suitable system for the detection of IF7 in the various chromatographic steps, showing stimulation by addition of the fractions coming from distinct purification steps. Having a system that was able to detect IF7 in an assay *in vitro*, a new attempt for the purification of this factor could be started.

### 3.3 IF7 purification from HeLa cells

IF7 was purified according to the scheme presented on Figure 3.5 (section 2.5.2) and the activity of the various fractions assessed using the above described fractionated system. At each stage, protein fractions were mixed with nA, nB, Q5 and H5 fractions and the mixture assessed for the ability to promote *in vitro* DNA repair synthesis in UV-damaged DNA. Activity that could stimulate damage-dependent repair synthesis by the nA, nB, Q5 and H5 fractions would be isolated and further purified. IF7 stimulatory activity was found to bind phosphocellulose, hydroxyapatite and ssDNA cellulose columns (Fig. 3.5).
As an example, the activity assay for fractions from the dsDNA cellulose column is shown in Figure 3.6. Reactions in lanes 6 to 9 all contained nA, nB, Q5 and H5 and are supplemented with different column fractions derived from the second step of this purification procedure, an hydroxyapatite column. In lane 6, ssDNA cellulose fraction 0.6 M KCl was tested for its activity. This fraction had been previously isolated from step 3 and loaded on a dsDNA cellulose column. The three fractions from this column, eluted at 0.1 M KCl, 0.3 M KCl and 0.6 M KCl were added to the next three lanes in this assay (Fig. 3.6).
Fig. 3.6 - Activity of IF7 purification fractions in repair synthesis reconstituted with nA, nB, Q5 and H5. UV-irradiated and non-irradiated plasmids were incubated with fractionated HeLa cell extracts as described (section 2.14.4); reactions contain 12 μg nA (or 500 ng RPA plus 60 ng PCNA), 40 μg nB, 21 μg Q5, 3.5 μg H5 and 6 μg H2+H3, or 5 μl ssDNA or dsDNA column fractions as indicated. H2 and H3 are the fractions containing IF7 from the HeLa fractionation procedure.
Fig. 3.7 - Purified IF7 from HeLa cells - repair activity in fractions from the Superose 6 column.

(A) Peak fractions from the Superose 6 column were tested for repair activity on a repair synthesis assay; reactions contain 12 μg nA, 40 μg nB, 21 μg Q5, 3.5 μg H5 and 5 μl of each of the indicated Superose 6 fraction.

(B) The peak fractions (20 μl of each) of the last column, Superose 6, were run on a 12% SDS-PAGE and silver nitrate stained; the fraction containing IF7 activity is fraction 17.
IF7 stimulatory activity peaked in fraction dsDNA0.3 (Fig. 3.6 lane 8) and therefore this was the fraction that was loaded onto a MonoQ column for further purification. The flow-through fraction from the MonoQ chromatographic step was loaded on a MonoS column and the active fraction from this column (0.3 M KCl) used for further analysis by gel filtration.

Peak fractions from the last step of this procedure (Superose 6 gel filtration chromatography) were likewise tested for their activity by addition to the fractionated system, and IF7 stimulatory activity found in fraction 17 (Fig. 3.7A). Fractions 13 to 23 were analysed by electrophoresis on a 12% polyacrylamide SDS gel and staining with silver nitrate (Fig. 3.7B). By SDS-PAGE analysis of the fraction containing the activity (fraction 17), it was apparent that the band that best correlated with IF7 was the one of relative molecular mass about 60 kDa. By gel analysis, this band was estimated to have a concentration of about 10 μg/ml. Therefore, the total protein obtained from this preparation was predicted to be approximately 1 μg. An attempt was made to microsequence this protein in collaboration with Dr. Darryl Pappin (ICRF, Lincoln's Inn Fields, London). Unfortunately these amounts were not enough for microsequencing the polypeptide and more detailed information on this factor could not be obtained.

3.4 IF7 activity in NER in vitro

IF7 was purified as described and its activity tested on a HeLa fractionated system where it acted as a stimulatory factor. However, it was necessary to test for the involvement of this new protein factor in a more defined NER system in vitro. To this end, IF7 activity was tested in NER reconstituted in vitro with purified proteins, using UV-damaged and non-damaged DNA substrate plus purified components for the incision/excision step, and exo-free pol I for the synthesis step (Fig. 3.8). In the absence of IF7, the characteristic DNA synthesis in both damaged and undamaged plasmids could be observed as previously...
(Fig 3.8 lanes 5-7). However, when IF7 was added to the reactions, exo-free pol I synthesis was performed in a damage-specific manner (Fig 3.8 lanes 1-4) further confirming the presence of this factor after the 7th purification step. The TFIIH fraction used during the study performed by Aboussekhra et al. was highly purified after six purification steps (TFIIH Hap, see Fig. 4.1). When replication proteins were used to perform the synthesis step, these reactions were completely dependent on IF7, not showing any damage dependent synthesis in the absence of this factor, whereas non-specific DNA synthesis was seen when exo-free pol I was used (Aboussekhra et al. 1995). The same was seen with this IF7 preparation purified from HeLa cells, both in the presence of TFIIH Hep and the more pure TFIIH Hap. In the absence of IF7, there was a non-specific incorporation of radiolabel that disappeared upon IF7 addition (Fig. 3.8 compare lanes 4 with 5 and 3 with 7).

A reconstituted system can be used in an incision assay developed J. G. Moggs that detects the excised 24-32 nucleotide long damaged oligomer by Southern blot hybridisation with a probe complementary to that region (section 2.14.2 and (Moggs et al. 1996; Shivji et al. 1998b)). When NER was reconstituted with purified components and the excised products detected by Southern hybridisation, IF7 had a stimulatory effect (Fig. 3.9). In this case, IF7 stimulates the incision/excision about 3-fold (Fig. 3.9 lanes 1 and 2) and again was not absolutely required. In the absence of ERCC1-XPF no incised products were detected and the same was observed when TFIIH Hap was used, perhaps due to the low NER activity of these highly purified fractions (see also Figs. 3.8 and 3.10 and chapter 4).
Fig. 3.8 - IF7 activity in a repair synthesis assay reconstituted with purified proteins and exo-free pol I. UV-irradiated and non-irradiated plasmids were incubated with purified protein fractions as indicated; each 30 µl reaction contains: 500 ng recombinant RPA, 45 ng recombinant XPA, 50 ng recombinant XPG, 12 ng of HeLa purified XPC complex and 1 µl of HeLa purified ERCC1-XPF complex; when indicated reactions contained 3 µl IF7; volumes of TFIIH complex are as indicated; repair synthesis was performed by exo-free pol I.
Additionally, IF7 activity could be tested in a dual incision assay where
the excised oligomers are detected directly by end labelling (section 2.14.1). This
method is more sensitive than the detection by Southern hybridisation, being
more suitable for testing the dual incision activity characteristic of NER of both
protein mixtures and cell extracts. As already seen by Southern blot analysis,
addition of IF7 to reactions performed in the presence of TFIIH Hep stimulates
dual incision formation about 3-fold (Fig. 3.10A lanes 2 and 4). In this assay,
addition of IF7 to a NER reaction complemented the repair activity of TFIIH
Hap (Fig. 3.10B lanes 3 and 4), the most purified HeLa TFIIH (see Fig. 4.1 and
chapter 4). TFIIH Hap showed no activity in the absence of IF7, in contrast with
both TFIIH Hep and SD fractions which were less purified (see chapter 4).
Obviously, this could be due only to the fact that IF7 activity is stimulatory,
perhaps the TFIIH Hap fractions had such a low activity that dual incisions
could only be detected under IF7 stimulation. Alternatively, it was possible that
IF7 could be essential for NER in vitro in the presence of a highly purified
fraction of TFIIH meaning that TFIIH was only active because it contained IF7.

The two latter NER incision assays (Figs. 3.9 and 3.10) use a cisplatin
lesion, whereas the first repair synthesis assays were done in the presence of
UV-damaged DNA. However, in all assays we observe the same effect of IF7 -
stimulatory when TFIIH Hep is used and essential when TFIIH Hap is used
(Aboussekhra et al. 1995).

All this evidence led us to a more detailed study of TFIIH complex that
will be presented in the next chapters of this thesis (mainly Chapters 4 and 5).
Fig. 3.9 - IF7 activity detected by Southern Blotting Pt (GTG) single lesion DNA substrate was incubated with purified proteins as indicated and detection of dual incision products was done by Southern blotting; amounts of protein are the same as in Figure 3.8.
**Chapter III - IF7 purification**

**Fig. 3.10 - IF7 activity detected in dual incision assay.** Pt (GTG) single lesion DNA substrate was incubated with purified proteins as indicated and dual incision detected by direct end labelling of the excised products; amounts of protein per 8.5 µl reaction are as specified in section 2.14.1; TFIIH amounts are as indicated and 3 µl of IF7 were added when indicated; TFIIH Hep SD is a TFIIH complex purified over 4 columns (Chapter 4 - Fig. 4.1).
Whilst studying different TFIIH complexes that were purified by different procedures, the conditions for the dual incision assay were slightly changed. This eradicated the previously observed NER dependence on IF7 and came as a surprise. Under the new conditions TFIIH Hep and TFIIH Hap were equally active in NER and IF7 was not shown to have a stimulatory effect (Fig. 3.11). The difference between the two systems shown in Figures 3.10 and 3.11 is that in the latter a new buffer batch was used (named N5X to differentiate from old 5X). The old 5X buffer was present in all reactions described so far and contains 40 mM Hepes-KOH pH 7.8, 5 mM MgCl₂, 0.5 mM DTT, 12.5% glycerol (v/v), 2.5 μg BSA, 40 mM phosphocreatine and 2 mM ATP. The new buffer (N5X) has nominally the same composition. However, they gave different results (compare Figs. 3.10 and 3.11). With old 5X there was a dependence on IF7 during NER \textit{in vitro}, whereas the same did not happen when N5X was used.

In order to clarify the difference between these two buffers, titrations of some of the components of this buffer were done. Titrations of phosphocreatine, CPK, ATP, DTT and MgCl₂ were studied but none of these components seemed to be responsible for the difference in activity of these two buffers. In addition, each of the components was used to complement the activity of the old 5X. That is, reactions were performed with either TFIIH Hep or with TFIIH Hap in the presence of old 5X and each of the components of this buffer was added \textit{de novo} to the reaction. The reason for this procedure was to test if any of the components added fresh could rescue the NER activity of the reactions done with TFIIH Hap. The rationale behind it was that the difference between old 5X and N5X was due to one of the components having changed its activity over time and storage.
Chapter III - IF7 purification

RPA, XPA, XPC-hHR23B, XPG, ERCC1-XPF

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPA, XPA, XPC-hHR23B, XPG, ERCC1-XPF</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TFIH HEP 1.5</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFIH HEP 1.5</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IF7</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 3.11 - Comparison of TFIH Hep and TFIH Hap activities with and without IF7. Each reaction contains the amounts of pure proteins specified in section 2.14.1; reactions are performed with N5X (the more recent reaction buffer).
As shown in Figure 3.12, lanes 1 and 8 contain TFIIH Hep and TFIIH Hap, respectively, but NER activity is detected in lane 1 but not lane 8. Nevertheless, if N5X was used (Fig. 3.12 lanes 7 and 14), exactly the same fractions show a different result. Under the new reaction buffer both TFIIH Hep and TFIIH Hap were active in NER. In lanes 2-6 and 8-13 the different components of this new buffer were tested. All different components have different effects in the reactions, both inhibitory (MgCl₂, lane 2) and stimulatory (DTT, lane 3). However, none of these can complement the negative activity of TFIIH Hap which is rescued in the presence of the new buffer (Fig. 3.12 lane 14). Due to this evidence, it was concluded that most probably the old buffer was working under sub-optimal conditions, perhaps containing some inhibitory activity.

Different combinations of the NER reaction components were tested for their effect in the overall inhibition of reactions in the presence of TFIIH Hap, but not TFIIH Hep. Nothing was shown to have the same effect as the previously used reaction buffer. The conditions under which TFIIH Hap was inactive and able to be rescued by IF7 were not reproducible unless the old 5X buffer was used. The inhibitory properties of this buffer were clear and therefore the effect of IF7 on the in vitro NER reaction was to overcome this inhibition. For this reason, no more attempts were done to purify IF7, since its activity was shown not to be essential for NER in vitro.
Fig. 3.12 - Comparison of TFIIH Hep and TFIIH Hap activities under different condition buffers. Each reaction contains the amounts of pure proteins specified in section 2.14.1 and the specified concentration of the different reaction components; old 5X is the reaction buffer used in the previously shown reactions; N5X is the more recent reaction buffer; reactions 1-7 contain 1.5 μl of TFIIH Hep and reactions 8-14 contain 1.5 μl of TFIIH Hap; both 5X buffers are supplemented with the indicated additional amount of each ingredient.
3.5 Discussion

During this chapter attempts to purify a new factor involved in NER were described. The amount of protein purified over this 7 step purification procedure, was not enough for microsequencing, precluding definitive identification of the stimulatory factor. Since the protein activity was shown not to be essential for the NER reaction \textit{in vitro}, no more attempts were made to purify this factor.

There are, however, several clues to the identity of IF7, stemming either from the purification procedure or from its effect during \textit{in vitro} NER. From the characteristics of IF7 during purification we can observe binding to ssDNA cellulose (only eluting at 0.6 M KCl) and dsDNA cellulose (eluting at 0.3 M KCl). This suggests that this factor has DNA binding motifs and might bind DNA during NER or other cellular processes. In addition, the effect of IF7 during repair synthesis assays might suggest that it binds to DNA nicks in a manner that will inhibit the non-specific synthesis performed by exo-free pol I.

IF7 was an unidentified activity involved in NER \textit{in vitro}. Its effect could be detected by different methods under different forms. When added to a repair synthesis assay where exo-free pol I was present it would suppress DNA synthesis in the undamaged plasmid (Fig. 3.8). On the other hand, it was necessary when TFIIH Hap was used in a fully reconstituted repair synthesis assay (Aboussekhra \textit{et al.} 1995). Additionally, IF7 showed a 3-fold stimulation of NER in the presence of TFIIH Hep (Figs. 3.9 and 3.10). More interestingly, it was necessary for dual incision formation \textit{in vitro} in the presence of TFIIH Hap (the highly purified TFIIH from HeLa cells) but not TFIIH Hep. TFIIH Hep is purified from HeLa over 4 chromatographic steps (see Fig. 4.1), whereas TFIIH Hap is further purified on a phenyl and on a hydroxyapatite column, yielding a near homogeneous fraction. Clearly, the next step would be to investigate if IF7 co-purified with TFIIH up to the 4th purification step and then the two activities would separate as previously seen with IF7 and ERCC1-XPF (see
chapter 4). Yet, while this was being done, the utilisation of different conditions led to the conclusion that the reactions were previously being performed under sub-optimal conditions and therefore the stimulatory effect of IF7 could be seen. Under the new conditions, IF7 had no effect and TFIIH Hep and Hap were equally active (Fig. 3.12 and chapter 4). Consequently, no more attempts were made to purify IF7. Further studies on the minimal set of factors necessary for NER in vitro are presented in the next chapter.

So, what is IF7? It is a stimulatory factor for NER in vitro, needed for the reactions under sub-optimal conditions but not when a highly sensitive assay is utilised under optimal conditions. Taking all the evidence into account an hypothesis can be put forward. IF7 could have DNA binding activity that would mediate its function as a stimulatory factor by binding to DNA nicks and preventing their degradation or interference with the NER reaction. However, under optimal conditions the IF7 effect would not be detected because this background inhibition would be at a very low level comparable to the yield of the NER reaction. Several of stimulatory factors for the in vitro NER reaction exist. Most of them are only detected under sub-optimal conditions. For example, RPA is known to stimulate the repair of defective cell extracts and even of NER normal extracts (Coverley et al. 1991; Rapic-Otrin et al. 1998). In the next chapter, it is shown that a kinase inhibitor can stimulate the dual incision activity of both cell extracts and reconstituted reactions. However, for further investigations on IF7 to be carried out more reproducible conditions for its detection and study need to be found. Nonetheless a different approach could be used to investigate the involvement of IF7 in the in vitro NER reaction. Kinetic measurements of the NER reaction could be made both in the presence and in the absence of IF7. Perhaps this factor will only show its effect by changing the reaction rate.
4.1 Introduction

In human cells, the proteins necessary for the incision reaction include XPA, XPC-hHR23B, RPA, TFIIH, and the nucleases XPG and ERCC1-XPF (Aboussekhra et al., 1995; Mu et al., 1996; Mu et al., 1995). In S. cerevisiae, incision of UV lesions can be accomplished by the homologous set of proteins Rad14, Rad4-Rad23, RPA, TFIIH, and the nucleases Rad2 and Rad10-Rad1 (Guzder et al., 1995; He et al., 1996) (see Introduction).

Uncertainties remained as to whether this list of proteins represented the truly minimal set of polypeptides involved in incision (see chapter III). An additional factor in S. cerevisiae, comprised of the Rad7 and Rad16 polypeptides, was designated NEF4 (nucleotide excision repair factor 4). It significantly increases incision efficiency in the yeast system (Guzder et al., 1997) and may involve an ATP-dependent damage recognition system (Guzder et al., 1998; Guzder et al., 1997) or be necessary for post-incision events (Reed et al., 1998). Yeast cells with Rad7 or Rad16 mutations show very little repair of pyrimidine dimers in non-transcribed DNA strands (Verhage et al., 1994) and this complex is necessary for repair by yeast cell extracts in vitro (Wang et al., 1997). Also, disruption mutants of RAD7 and RAD16 have shown that these genes are responsible for an inducible component of NER that has been observed in S. cerevisiae (Scott and Waters, 1997; Waters et al., 1993). During reconstitution of NER with UV-irradiated DNA and using DNA pol I (exo-free pol I) to perform damage dependent DNA synthesis, an additional unidentified protein factor, designated IF7, that significantly stimulated repair was reported.
(Aboussekhra et al., 1995) (chapter III). Further, the UV-DDB factor, defective in XP group E cells, is important for full NER in vivo, particularly for repair of cyclobutane pyrimidine dimers in non-transcribed DNA (Hwang and Chu, 1993; Hwang et al., 1999; Reardon et al., 1993). It strongly binds DNA in vitro and was also shown to have a stimulatory effect on repair of UV irradiated DNA (Aboussekhra et al., 1995). Another factor with a marked effect on yeast NER in vivo is the MMS19 gene product which appears to activate the TFIIH component for repair (Lauder et al., 1996; Lombaerts et al., 1997). So far, experiments to reconstitute the NER incision reaction have used a mixture of recombinant components and proteins purified from cell extracts. The possibility still remained that some components co-purified with unidentified factors required for the core repair reaction.

A second outstanding question is which form of the transcription-repair complex TFIIH works best in repair. Mammalian TFIIH contains nine characterised subunits, of which three subunits form the cdk-activating kinase (CAK) complex (reviewed in (Moncollin et al., 1998; Svejstrup et al., 1996)). Evidence exists that in yeast there are interchangeable TFIIH forms with different principal tasks, one form containing CAK, that works mainly in transcription, and another without CAK components (core TFIIH) for repair (Svejstrup et al., 1995). Depending on the methods used to isolate the TFIIH complex from cells, differences in the subunit composition can be observed (Rossignol et al., 1997). Furthermore, a significant amount of kinase complex (CAK) may exist in free form in the cell (Adamczewski et al., 1996; Feaver et al., 1994; Rossignol et al., 1997).

In order to reach the most definitive conclusion on the minimal set of factors necessary for NER in vitro, I set out to reconstitute the dual incision reaction with exclusively recombinant, highly purified components. Advantage was taken of the recent availability of recombinant TFIIH by a collaboration with F. Tirode and J.-M. Egly in Strasbourg (Tirode et al., 1999) that allowed to assess the NER activity of both 6 and 9 subunit forms of
Chapter IV - Reconstitution of NER

TFIIH. Finally, these recombinant damage recognition and incision components were combined with highly purified human DNA synthesis proteins in order to completely reconstitute repair.

4.2 TFIIH purification

In the previous chapter, dual incision was reconstituted with a mixture of HeLa purified and recombinant factors. TFIIH was purified from human cells over a 4- or a 6-step purification procedure. During the course of this study, all incision factors, except TFIIH, became available in the recombinant form (Fig. 4.2). The reconstituted system was well defined and if any other factors were necessary for the dual incisions to occur in vitro they would have to be co-purifying with the multisubunit TFIIH complex.

TFIIH is purified from HeLa cells according to the scheme developed by J.-M. Egly and co-workers (Gerard et al., 1991; Marinoni et al., 1997), represented in Fig. 4.1A. In order to be able to investigate further the composition of this factor, I also purified TFIIH from HeLa cells according to the scheme represented in Fig. 4.1B.

The HeLa purified TFIIH fractions used in this study are:
- TFIIH Hep fraction IV, from Heparin HPLC column;
- TFIIH Phe fraction V, from Phenyl HPLC column;
- TFIIH Hap fraction VI, from Hydroxyapatite HPLC column;

all done in collaboration with J.-M. Egly's laboratory, Strasbourg, France.

The TFIIH purification I performed was used in order to analyse the differences in NER activity between TFIIH complexes purified further from the Heparin, step IV. TFIIH Hep SD (Fig. 4.1B) fractions were further purified by different chromatographic steps in order to test if any other factors necessary for the NER reactions were being separated from the multisubunit TFIIH. This stems from initial observations that TFIIH Hep
was fully active in NER whereas TFIIH Hap showed no activity and could be complemented by IF7 (see chapter III).

Fig. 4.1 TFIIH purification scheme.
A - TFIIH purification scheme from HeLa cells as performed in J.-M. Egly's laboratory; fractions from different steps are numbered from I to VI for clarity.
B - TFIIH purification scheme from HeLa cells done for co-purification studies; TFIIH from this purification procedure is designated TFIIH “SD”.

4.3 Reconstitution of dual incision with recombinant factors and TFIIH fractions purified from HeLa cells

To monitor the dual incision reaction of NER, a closed-circular plasmid containing a single 1,3-intrastrand d(GpTpG) cisplatin crosslink and a method that permits direct end-labelling of the damaged excised products were used (section 2.14.1). This DNA substrate was combined with the highly purified recombinant proteins shown in Fig. 4.2.
**Fig. 4.2 - Purified proteins used in in vitro reconstitution assays.**

**A** - Incision factors (top line) and repair synthesis factors (bottom line); XPA, RPA, XPG, PCNA and ligase I gels are Coomassie blue stained and all the others are silver stained; asterisks represent the various subunits of human RFC, pol δ and pol ε.

**B** - Recombinant TFIIH; silver stained gels (left) and immunoblots (right); equivalent amounts of rIIH6 and rIIH9 have been loaded in each panel.
Fig. 4.3 TFIIH active in transcription is also active in NER.
A - Upper panel: 5 μl of each step V fractions were run in a 10% SDS-PAGE and immunoblotted against the three subunits of TFIIH indicated. Lower panel: 3 μl of each step V fractions were used in a nucleotide excision repair assay containing all the recombinant proteins needed for dual incision formation (except TFIIH) - see Figure 4.2; TFIIH Hep fr. IV is 1.5 μl.

B - Upper panel: 5 μl of each step VI fractions were run in a 10% SDS-PAGE and immunoblotted against the three subunits of TFIIH indicated. Lower panel: 3 μl of each step VI fractions were used in a nucleotide excision repair assay containing all the recombinant proteins needed for dual incision formation (except TFIIH); TFIIH Hep fr. IV is 1.5 μl.
XPA, RPA, and ERCC1-XPF complex were produced in *E. coli* and XPG and XPC-hHR23B complex produced in insect cells as described ((Henricksen *et al.*, 1994; Jones and Wood, 1993; Masutani *et al.*, 1994; O' Donovan *et al.*, 1994; Sijbers *et al.*, 1996) and section 2.10).

In order to define the form of TFIIH needed for incision and to assess the need for any further incision proteins, these five recombinant factors were incubated with DNA containing a single defined Pt(GTG) lesion and TFIIH from HeLa cells, purified through various steps as described (Marinoni *et al.*, 1997). NER activity was followed by direct end-labelling of the nucleotides produced by dual incision. Throughout the purification procedure (outlined in Fig. 4.1A), TFIIH activity was followed by assaying for transcription initiation activity, helicase and ATPase activities and by immunoblotting for known TFIIH components (Gerard *et al.*, 1991; Marinoni *et al.*, 1997) (in J.-M. Egly laboratory).

TFIIH purified from HeLa cells over 4 chromatographic steps, TFIIH Hep fr. IV, was highly active in NER in combination with the other purified components (Fig. 4.3A, lanes containing Hep fr. IV and Load). This Hep fraction was subjected to two further HPLC purification steps, a phenyl-5PW and a hydroxyapatite column (Fig. 4.1A). TFIIH fractions purified over 5 chromatographic steps, TFIIH Phe fr. V, are active in transcription and in dual incision formation. The peak of protein, detected by immunoblotting, coincides with both activities - NER (Fig. 4.3A fr. 16-30) and transcription (J.-M. Egly, personal communication).

The fraction V TFIIH was further purified through a hydroxyapatite column, yielding a highly purified TFIIH Hap fraction VI (Fig. 4.1 and 4.2). NER activity of fraction VI also peaked in the same fraction as transcription activity (Fig. 4.3B fr. 6).
**Fig. 4.4 - TFIIH Hap is free of other NER factors.** NER was performed in the absence of each of the repair factors as indicated in the figure and in the presence of 2 µl TFIIH Hap fr. VI (lanes 2-7), 1.5 µl TFIIH Hep fr. IV (lane 1) and 2µl TFIIH Hap fr. VI from a previous purification procedure (lane 8); TFIIH Hap fr. VI is fraction 6 from Fig. 4.3B.
Individual omission of any of the other factors (Fig. 4.4) abolished repair (lanes 3-7 of Fig. 4.4 show no dual incision activity when any of the other NER factors is absent), showing that the reaction is totally dependent on the addition of RPA, XPA, XPC-hHR23B complex, XPG, ERCC1-XPF and TFIIF and that these repair factors do not cross-contaminate TFIIF Hap fraction VI.

4.4 Reconstitution of dual incision with recombinant TFIIF

The above results do not completely rule out the possibility that another polypeptide is needed for the reaction and co-purifies with TFIIF Hap fr. VI. TFIIF purified by this method still contains some unknown polypeptides, detectable by silver staining of SDS-PAGE gels (Fig. 4.2), and several studies outlined in section 4.1 have suggested that additional factors might be involved.

To further define the peptides involved in the dual incision stage of NER, two different recombinant TFIIF complexes were produced in insect cells by J.-M. Egly's laboratory in Strasbourg. A 9-subunit TFIIF containing XPB, XPD, p62, p52, p44, p34, cdk7, cyclin H and MAT1 was designated rIIH9 and a TFIIF lacking the latter three CAK subunits was designated rIIH6 (both complexes were His-tagged on p44 and cyclinH) (Tirode et al., 1999). Both complexes were purified over a heparin-sepharose column and a metal chelate affinity column and are active in transcription, rIIH9 having higher activity than rIIH6 due to the presence of the CAK subcomplex (Tirode et al., 1999).

Both rIIH6 (Fig. 4.5 lanes 4-6) and rIIH9 (Fig. 4.5 lanes 7-9) were found to be active in the first stages of NER. rIIH6 appears to be slightly more active than rIIH9 even though the concentration of these two complexes is approximately the same as measured by immunoblot and silver staining of equivalent volumes (Fig. 4.2 and F. Tirode, personal communication).
Fig. 4.5 - Recombinant TFIIH is active in NER. Different amounts of rIIH6 and rIIH9 were used in a nucleotide excision repair assay containing all the recombinant proteins needed for dual incision formation, as indicated; TFIIH Hep fr. IV is 1.5 μl; HeLa is 20 μg of whole cell extract protein. Quantification was done on a phosphorimager (imageQuant), where the density of the bands corresponding to the 26-mer to 30-mer excision products (migrating as 30-34-mers on this gel because the direct labelling procedure adds 4 residues) was quantified, these values were divided by the value corresponding to the reactions performed in the presence of TFIIH Hep fr. IV to give "relative counts" plotted in the graph.
These results show that although TFIIH containing the CAK subcomplex functions in repair, it is not needed for dual incision formation in vitro. The response of the reaction appeared to be rather non-linear in terms of the amount of TFIIH added (Fig. 4.5). But the high activity attained with rIIH6 raised the possibility that the presence or activity of the CAK subunits might have an inhibitory effect on the NER reaction.

4.5 Effect of CAK and CAK inhibitors in NER

To directly pursue the influence of CAK subunits in NER, recombinant CAK complex (rCAK) was produced in insect cells as described (Rossignol et al., 1997) in J.-M. Egly's laboratory. The activity of TFIIH complexes containing 6 subunits (rIIH6) with and without the addition of rCAK was then tested in a dual incision assay (Fig. 4.6). Under the conditions used in this assay, we could not detect any differences in the NER activity of TFIIH upon adding CAK (Fig. 4.6 compare lanes 3-5). It was noticeable, however, a significant effect of CAK when an ATP regenerating system (consisting of phosphocreatine and creatine kinase) was included in the reaction mixture (Fig. 4.6 lanes 6-9). This system is known to replenish the ATP concentrations at the same time they are used (reviewed in (Lyzlova and Stefanov, 1991)). In this case, addition of CAK to rIIH6 inhibited the reconstituted NER reaction (Fig. 4.6 compare lane 6 with 7, 8). This suggested that the higher steady state levels of ATP achieved by inclusion of an ATP-regenerating system could lead to an inhibitory effect of CAK kinase by phosphorylating some component of the reaction.

Using these conditions a known chemical inhibitor of CAK kinase activity was checked - the isoquinoline sulfonamide derivative H-8. H-8 inhibits the kinase activity of cdk7 and its phosphorylation of the RNA polymerase II CTD during transcription (Dubois et al., 1994).
Fig. 4.6 - CAK inhibits dual incision formation in the presence of high levels of ATP.

rIIH6 (2 µl) was tested in a dual incision assay in addition to increasing amounts of rCAK in the presence (lanes 6-9) or in the absence (lanes 1-5) of an ATP regenerating system constituted by phosphocreatine and creatine phosphokinase; the effect of H-8 in the presence of rCAK was done by addition of this inhibitor to a final concentration of 100 µM (lane 9). Relative counts are calculated using the NER activity of TFIIH Hep fr. IV as 1.
Fig. 4.7 - H-8 stimulates dual incision formation in the presence of high levels of ATP and TFIIH Hep. TFIIH Hep fr. IV (1.5 μl) was tested in a dual incision assay, either in absence (lanes 1-5) or in presence (lanes 6-10) of an ATP regenerating system and addition of H-8 was done as indicated.
Fig. 4.8 - H-8 stimulates dual incision formation in the presence of high levels of ATP and TFIH Hap.
TFIH Hap fr. VI (1.5 µl) was tested in a dual incision assay in presence of an ATP regenerating system (as in Fig. 4.7) and addition of H-8 was done as indicated.
Fig. 4.9 - TFIIH without the CAK subunit is not inhibited by CAK. TFIIH without the CAK subunit (TFIIH (-CAK) - 2 μl) purified from HeLa cells, was tested in a dual incision assay, in the presence of the same ATP regenerating system as in B and C, where increasing amounts of the kinase inhibitor H-8 were added as indicated (lanes 4-6); 1 μl rCAK was added to the reaction in the absence of H-8 (lane 7); quantification was performed as in Fig. 4.5 in order to give "relative counts" plotted in the graph.
H-8 was used in dual incision assays with various TFIIH fractions all in the presence of ATP regenerating system. Addition of H-8 to reconstituted reactions containing TFIIH Hep fr. IV increased its activity in NER by about 3-fold (Fig. 4.7 lanes 7-10). In reaction mixtures with the purest TFIIH fraction from HeLa (TFIIH Hep fr. VI), addition of increasing amounts of H-8 could stimulate repair by up to 5-fold (Fig. 4.8 lanes 2-5). Further, addition of 100 μM H-8 to reactions containing rIIH6 and rCAK overcame some of the inhibition caused by addition of the kinase complex in the presence of phosphocreatine and creatine phosphokininase (Fig. 4.6 lane 9). There was no effect of H-8 on the NER activity when TFIIH Hep fr. IV complexes were used in reaction mixtures without an ATP regenerating system (Fig. 4.7 lanes 1-5). When TFIIH was used that was depleted of its CAK subcomplex (TFIIH(-CAK)) (Rossignol et al., 1997) H-8 did not stimulate repair, even in the presence of an ATP regenerating system (Fig. 4.9). Taken together these results show that NER activity can be partially inhibited by CAK if ATP is kept at a high level.

4.6 Complete reconstitution of repair synthesis of a single lesion substrate with purified human factors

Nucleotide excision repair synthesis was previously reconstituted in vitro using multiple lesion UV-damaged plasmid DNA substrates and a mixture of proteins purified from HeLa cells, bacteria, and insect cells, and DNA polymerase components purified from calf thymus (Aboussekhra et al., 1995; Shivji et al., 1995). It was found that calf thymus DNA polymerase ε could function in repair in conjunction with the recognition-incision proteins (Aboussekhra et al., 1995). The availability of the highly purified recombinant incision system described above allowed us to determine if these proteins could be combined with DNA polymerase components in order to obtain full repair.
Fig. 4.10 - Reconstitution of repair synthesis with recombinant and human purified factors.

A - Schematic representation of the DNA substrate containing a single 1,3-intrastrand d(GpTpG) cisplatin crosslink with the sizes of the fragments generated by BstNI restriction digestion shown underneath; the arrows indicate mapped positions of incisions (Moggs, et al 1998)

B - Reconstitution of repair synthesis on a single lesion plasmid; dual incision reactions were performed by RPA, XPA, XPC-hHR23B, XPG, ERCC1-XPF and TFIIF Hep where indicated or by RPA and CFII; after incisions, synthesis was performed by addition of PCNA (lane1), exo-free pol I (lanes 2 and 3) and PCNA, polymerase δ or ε (as indicated), RFC and DNA ligase I.
Instead of using the UV-irradiated DNA described previously, I used the DNA containing a single specifically located cisplatin lesion, Pt(GTG). For DNA polymerase components only highly purified human enzymes instead of components from calf-thymus were used. Finally, systems were checked using both DNA polymerase δ and ε, as gap-filling studies suggested that either polymerase could work for synthesising DNA in NER generated incision gaps (Shivji et al., 1995; Wood and Shivji, 1997).

The repair synthesis assay used allows following specific repair synthesis of a defined adduct. For this purpose, the same damaged DNA molecule was used as in the incision assays presented above, but reaction mixtures included a $[^{32}P]$-deoxynucleotide so that patches would be radiolabelled during repair synthesis. Cleavage of the closed circular M13 molecule containing the cisplatin adduct with BstNI restriction enzyme generates a 33 nucleotide fragment that includes the repair site, and several larger fragments (Moggs et al., 1996). Synthesis arising specifically from filling the 24-32 nt gap during NER should be largely confined to the 33 nt fragment (labelled "C" in Fig. 4.10A), with some specific synthesis in the 68 nt fragment (labelled "B" in Fig. 4.10A) because some mapped 5' incision sites fall within this fragment (Fig. 4.10A).

First, reconstitution experiments were carried out in two stages (i) dual incision/excision and (ii) repair synthesis. The reaction mixtures included recombinant RPA, XPA, XPC-hHR23B, XPG, ERCC1-XPF, and TFIH Hep to carry out the dual incisions. Recombinant PCNA (Shivji et al., 1992) and ligase I (Mackenney et al., 1997), in addition to purified human RFC and polymerase δ or ε (Fig. 4.10 lanes 4-7)) or exo-free pol I (Fig. 4.10 lanes 2 and 3) were used to perform the synthesis step. As a positive control, dual incision reactions were also accomplished by recombinant RPA and the crude fraction CFII (HeLa whole cell extract after fractionation on phosphocellulose to retain RPA and PCNA (Shivji et al., 1992; Wold and Kelly, 1988)) (Fig. 4.10 lane 1) and the synthesis step accomplished by
addition of PCNA. CFII fraction contains all the factors necessary for repair synthesis except RPA and PCNA. Adding RPA to a CFII fraction will allow this reaction to perform dual incision and by further addition of PCNA this mixture will be able to fill in the gap generated by dual incision/excision (Shivji et al., 1992).

Both pol δ and pol ε could function in repair. The levels of synthesis observed with the two human polymerases were higher than with exo-free pol I and were specifically dependent on the addition of TFIH (Fig. 4.10 lanes 4-7).

In order to analyse only the synthesis step, repair intermediates were prepared. Single lesion substrate DNA, Pt(GTG), was incubated with RPA and CFII fraction in order to allow the incisions to occur. In the absence of PCNA, incision and excision of a damaged oligonucleotide can occur, but repair synthesis by the PCNA dependent DNA repair polymerase is prevented (Shivji et al., 1992; Shivji et al., 1995). After incisions, gapped DNA molecules were purified by phenol-chloroform extraction and ethanol precipitation and then used as substrate for gap-filling by DNA polymerase δ or ε (Fig 4.11). DNA polymerase ε and δ as well as exo-free pol I, CFII+RPA+PCNA and a XPA defective cell extract (XP2OS) can specifically synthesise DNA on the gapped repair intermediate (Fig. 4.11). DNA polymerase ε is dependent on PCNA and RFC as can be observed by the lack of repair products when RFC and PCNA are absent (Fig. 4.11 lanes 7, 8). Human purified RFC has an activity comparable to calf-thymus purified RFC (cRFC - Fig. 4.11 lanes 3-6). Human pol δ has an activity comparable to calf thymus pol δ (Fig. 4.11 lanes 10, 11) and to human pol ε (Fig. 4.11 lanes 5 and 11). These results demonstrate that human polymerases δ and ε and accessory factors can fill in the gap generated by dual incision/excision of a DNA lesion in vitro.
Fig. 4.11- Reconstitution of repair synthesis with recombinant and human purified factors. Reconstitution of repair synthesis on a single lesion plasmid; dual incision reactions were performed by RPA and CFII; after incisions, DNA was purified and synthesis performed by addition of RPA+PCNA+CFII (lane 1), exo-free pol I (lane 2), polymerase δ (lanes 3-9), polymerase ε (lanes 10 and 11) or XP-A extract; addition or omission of other factors is as indicated; cRFC - calf thymus RFC; RFC - human purified RFC; Pol δ “c” - calf thymus Pol δ; Pol δ “h” - human purified pol δ.
Results present so far indicate that repair synthesis by purified human DNA polymerases can take place on a repair intermediate that is the result of dual incision and DNA purification (Fig. 4.11) and additionally, human purified polymerases can act simultaneously to the incision step when TFIIH Hep is used (Fig. 4.10). In the next step repair synthesis was tested in reactions where the incisions were being performed by recombinant factors. Reconstitution was carried out with all the proteins required for NER in the same reaction mixture (12.5 µl volume) and results are presented in figure 4.12.

Activity was observed with TFIIH purified from HeLa cells (Fig. 4.12 lanes 2, 3 and 9, 10) as well as with 6-subunit recombinant TFIIH (lanes 6, 13) and 9-subunit recombinant TFIIH (lanes 7, 14). No repair was observed when TFIIH was omitted (Fig. 4.11 lanes 1 and 8). Under these conditions (approx. 70 mM salt) both the pol δ and pol ε reactions were dependent on PCNA (Fig. 4.11 lanes 5, 12) and the ATP-dependent PCNA loading factor RFC (Fig. 4.11 lanes 4 and 11). Both purified human DNA polymerases were able to fill in the gap produced by only recombinant incision proteins.

Taken together these results show that repair synthesis by DNA polymerase δ or ε can take place in a DNA molecule where the incisions have been placed by a full set of recombinant factors.
Fig. 4.12 - Reconstitution of repair synthesis with recombinant and human purified factors. Reconstitution of repair synthesis on a single lesion plasmid; different TFIIH preparations were used in reactions performed in the presence of polymerase δ (lanes 1-7) or ε (lanes 8-14); lanes 2 and 9 contain 1.5 μl TFIIH Hep fr. IV, lanes 3-5 and 10-12 contain 3 μl TFIIH Hap fr. VI, lanes 6 and 13 contain 3 μl rIIH6 and lanes 7 and 14 contain 3 μl rIIH9; reactions in lanes 3 and 11 are done in the absence of RFC and reactions in lanes 5 and 12 are done in the absence of PCNA.
4.7 Discussion

4.7.1 Dual incision and repair synthesis with purified human proteins

In order to unequivocally identify the proteins involved in the dual incision formation characteristic of NER, this reaction was reconstituted \textit{in vitro} using exclusively recombinant proteins. Since only six factors were used to reconstitute the first steps of the nucleotide excision repair reaction, the possibility of the existence of another protein(s) necessary for this reaction \textit{in vitro}, can be ruled out. Addition of purified IF7 to the reactions performed in the presence of the 5 recombinant factors and HeLa purified TFIIH (TFIIH Hep fr. IV or TFIIH Hap fr. VI) is neither stimulating nor inhibiting (chapter III). Recombinant RPA, XPA, XPC-hHR23B, XPG and TFIIH complex containing only 6 subunits are essential and sufficient for the reconstitution of the first stage of NER and can therefore be considered the "minimal set of factors" necessary for dual incision formation on naked DNA \textit{in vitro}. These comprise 15 or 18 polypeptides, depending on whether TFIIH with CAK subcomplex is used. Of course this does not rule out the participation of other stimulatory proteins which are likely to be needed in cells, such as Mms19, UV-DDB, homologues of Rad7 and Rad16, or enzymes yet to be identified. Some factors may, for example, assist with damage recognition in chromatin.

In addition, for the first time nucleotide excision repair synthesis was reconstituted using only highly purified human replication proteins and recombinant incision factors. Repair synthesis was performed on a single lesion DNA substrate by highly purified human DNA polymerase δ or ε, purified human RFC and recombinant RPA, PCNA and DNA ligase I. Overall the full repair reaction (dual incision + repair synthesis) can be reconstituted \textit{in vitro} by 10 components: RPA (involved in both steps), XPA, XPC-hHR23B, XPG, ERCC1-XPF, TFIIH complex (with 6 or 9 subunits), DNA
polymerase δ or ε, RFC, PCNA and DNA ligase I (and DNase IV when reactions included DNA polymerase δ). In total, only 26 polypeptides can perform the complete NER reaction on a naked damaged DNA substrate. Repair synthesis occurred on a single lesion DNA substrate with all proteins in the same reaction mixture, indicating that no additional factor is required for coupling dual incision to repair synthesis.

Repair synthesis under these reaction conditions depends on PCNA for both DNA polymerases δ and ε, as expected from previous studies with model DNA substrates containing short gaps (Podust and Hübscher, 1993; Shivji et al., 1995). With both polymerases, the RFC complex, which loads PCNA onto DNA in an ATP-dependent manner, was also required for repair synthesis on this circular template (Fig. 4.12). This was the first time that RFC dependence was directly demonstrated for NER.

4.7.2 Activity of TFIIH with and without CAK subunits in NER

When added to 5 recombinant factors, the TFIIH complementing activity for NER co-purifies as a single peak with the transcription activity (Fig. 2). This suggested that the same form of TFIIH can function in both transcription and in NER. In vitro, the transcriptionally active form of TFIIH is also NER active, in contrast with what was proposed in S. cerevisiae where there was evidence for a transcriptionally active TFIIH (holo-TFIIH) that would be active in transcription and a TFIIH without the CAK subcomplex that would form the "repairosome" and be active in NER (Svejstrup et al., 1995). CAK is dispensable for NER in vitro, but TFIIH containing this kinase subcomplex is still active in dual incision formation, as seen by using highly purified TFIIH from HeLa or the recombinant 9 subunit complex.

The physical presence of CAK is not inhibitory, but in presence of an ATP regenerating system CAK can inhibit the repair reaction in vitro.
Consistent with this effect, inhibition of CAK activity by H-8 kinase inhibitor could stimulate repair reactions or partially reverse the inhibitory effect of CAK. It seems that when the ATP levels are maintained at a high level the kinase in CAK has an inhibitory effect. Perhaps CAK is phosphorylating some component of the reaction, therefore rendering it inactive or lowering its activity. Preliminary results show that XPG is easily phosphorylated and a possible target of this kinase activity (S. J. Araújo and R. Ariza, unpublished). In general, these results are in accordance with previous observations that inhibition of phosphatase activity in cell extracts, in the presence of an ATP regenerating system, inhibits repair (Ariza et al., 1996). It is noteworthy that the kinase activity of TFIIH decreases after UV-irradiation of mammalian cells, suggesting that cells may have a mechanism to suppress CAK activity when DNA repair is urgently required (Adamczewski et al., 1996). Phosphorylation might be modulating the NER reaction both \textit{in vitro} and \textit{in vivo} and this deserves further investigation.

So, the question remains of what form of TFIIH is the most physiological form. Different forms of TFIIH have been detected in human cells, mostly by their different purification profiles. TFIIH can be resolved into four subcomplexes: CAK, core TFIIH (containing XPB, p62, p52, p44 and p34) and XPD-CAK or XPD-core TFIIH, in addition to the 9 subunit TFIIH (Drapkin et al., 1996; Reardon et al., 1996; Rossignol et al., 1997). However, Roy et al. observed that microinjection of an anti-cdk7 antibody into the cytoplasm of normal human fibroblasts could inhibit both transcription and NER hinting on the involvement of a 9-subunit TFIIH in both processes or that the antibody inhibits conversion into a form active in repair (Roy et al., 1994; Svejstrup et al., 1995). Since we have demonstrated that both 9 subunit TFIIH and XPD-core TFIIH are active in the formation of dual incisions, it remains to be demonstrated which is the actual form of this complex, performing NER \textit{in vivo}. In chapter VI, an immunoprecipitation method is
used to isolate TFIIH from HeLa cell extracts and essentially all the TFIIH detected is in the 9 subunit form.
CHAPTER V
Comparison of the effects in NER of XPD mutations

5.1 Introduction

The XPD gene encodes for a 5'→3' helicase, containing 7 distinctive helicase motifs, which is a component of transcription/repair factor TFIIH. Patients with mutations in this gene have one of three different clinical disorders: XP group D (XP-D), trichothiodystrophy (TTD) or a combination of XP and Cockayne syndrome (XP-CS) (see Introduction). The clinical features of XP, TTD and CS are quite distinct and it has been a puzzle as to how mutations in the same gene can give rise to such different phenotypes.

Several studies have tried to clarify this phenotype-genotype relationship by identification of the mutations involved in each syndrome (Broughton et al. 1994; Takayama et al. 1996; Taylor et al. 1997). In the study performed by Taylor et al., different mutations in the XPD gene were found to be associated with either XP or TTD. This was accomplished by examining the complementation phenotype in Schizosaccharomyces pombe cells of mutations analogous to those shared between TTD and XP. When different mutations were found in the two alleles, their behaviour was tested in haploid S. pombe cells. Homologous mutations were generated in rad15 (the S. pombe homologue of XPD) and the ability of these individual alleles to rescue the inviability of a rad15 null mutation was assessed. If a determined mutation behaved as a null in this system, the phenotype was most probably determined by the other allele and therefore the causative mutation could be determined (Taylor et al. 1997). By making use of the sequence conservation of XPD, different mutations in this gene could be attributed to different positions within the protein, correlating the site of the mutation with the clinical phenotype. Distribution of mutations reveals that
essentially most mutations that cause a XP-D or TTD phenotype are clustered within the C-terminal third of XPD (Taylor et al. 1997; Cleaver et al. 1999). However, this study did not delineate discrete domains in the primary structure of the protein particularly associated with the different phenotypes of XP or TTD. Perhaps the causative mutations are located in different physical areas of the protein but this hypothesis can only be tested when the three-dimensional structure of XPD is determined.

It has been suggested that XP-D results from mutations that influence only DNA repair, whereas TTD results from mutations that subtly affect transcription (Bootsma and Hoeijmakers 1993; Takayama et al. 1996; Taylor et al. 1997). This seems to indicate that the helicase activity of XPD is more important for NER, whereas mutations in the C-terminal domain of the protein cause TTD by affecting transcription, perhaps by interfering with interactions between XPD and other TFIIH subunits (Coin et al. 1998b).

Independently of which clinical phenotype they cause, mutations in XPD lead to UV sensitivity that does not correlate with the extent of unscheduled DNA synthesis (UDS) detected (Johnson and Squires 1992; Cleaver et al. 1999). Measurements of UV-induced UDS in cells with mutations in XPD show values around 20-50% of the normal level (Lehmann and Stevens 1980; Paterson et al. 1987; Johnson and Squires 1992; Broughton et al. 1995; Takayama et al. 1996). In contrast, extremely NER defective and UV sensitive XPA deficient cells have low levels of UDS (below 5%) (Cleaver and Kraemer 1995). It is normally assumed that UDS levels correlate with repair synthesis since they are a measure of DNA synthesis in non-S-phase cells. Furthermore, XP-D fibroblasts exhibit intermediate levels of nuclear accumulation of PCNA, in a study that directly correlated repair incisions with PCNA immunostaining levels (Aboussekhra and Wood 1995).

The high UDS levels of XP-D cells could be the result of DNA incision without subsequent repair. Any cleavage of the DNA chain, coming from
abortive repair events, could lead to unscheduled DNA synthesis. This would be UV-dependent but not a result of UV-repair, thus explaining the accompanying UV-sensitivity.

In this chapter, XPD activity in NER is analysed and the biochemical defects associated with the loss of its enzymatic activity discussed. Dual incision formation and uncoupled incision formation in 5 different XP-D cell lines is compared. Additionally, the NER activity and pattern of incisions performed by a purified TFIIH containing mutant XPD is investigated.

5.2 Cell lines with mutant XPD protein and their activity in dual incision

In collaboration with Mark Berneburg and Alan R. Lehmann (MRC-CMU, Sussex) different cell lines from patients with XPD mutations were analysed.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation (amino acid)</th>
<th>% UDS</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>XP8BR</td>
<td>G675R*</td>
<td>30-40%</td>
<td>(Broughton et al. 1995) (A.R. Lehmann, unpublished)</td>
</tr>
<tr>
<td>XP12BR</td>
<td>R511Q</td>
<td>30-40%</td>
<td>(A.R. Lehmann and B.C. Broughton, unpublished)</td>
</tr>
<tr>
<td>TTD5BR</td>
<td>A594P* splice mutation-intron 7</td>
<td>30%</td>
<td>(Motley and Finlay 1989) (A.R. Lehmann, unpublished)</td>
</tr>
<tr>
<td>XP1NE</td>
<td>L461V, Del (716-730) G47R*</td>
<td>40%</td>
<td>(Lehmann and Stevens 1980; Johnson and Squires 1992; Taylor et al. 1997)</td>
</tr>
</tbody>
</table>

Table 5.1 - Mutations in XPD gene in the XPD and TTD patient cell lines used in this study. Mutations are shown for both alleles and the causative mutation marked by *; in the case of XP12BR, the patient is homozygous for the mutation shown.
In order to study the NER activity of XP-D mutant cells, five WCEs of different cell lines established from patients of either XP group D, TTD or XP/CS were made, according to section 2.2. All cell lines show high levels of UDS (Table 5.1).

Three cell lines belong to XP group D (XP7BE, XP12BR and XP1NE) (Taylor et al. 1997), one to TTD (TTD5BR) (A. R. Lehmann, unpublished) and the other to XP/CS (XP8BR) (Broughton et al. 1995). The mutations present in these XPD alleles have been identified (Table 5.1 and Fig. 5.1) and the causative mutation has been detected using *S. pombe* cells (Taylor et al. 1997) (A. R. Lehmann, unpublished). Of these XP-D cell extracts, the only that has been biochemically studied in some detail is XP7BE (GM2485) (Evans et al. 1997b).

The NER activity of XPD mutants was tested by dual incision assay (section 2.14.1). Dual incisions were formed by TTD5BR (Fig. 5.2A lanes 2-3 and C lanes 6-7) and XP1NE (Fig. 5.2C lanes 9-10) but not by XP8BR (Fig. 5.2A lanes 7-8 and C lanes 2-4), XP7BE (Fig. 5.2A lane 16 and B lanes 9-11) or XP12BR (Fig. 5.2B lanes 2-4). All cell extracts could be complemented by either HeLa purified TFIIH (TFIIH Hep - Chapter 4) or by cross-complementation with CHO 43-3B WCE (defective in ERCC1). Although TTD5BR and XP1NE exhibited some dual incision activity, they showed further stimulation upon addition of complementing activity (Fig. 5.2A lanes 2-6 and Fig. 5.2C lanes 6-11).

![Fig. 5.1 - Mutations in the XPD protein](image)

Fig. 5.1 - Mutations in the XPD protein. Diagram of the XPD protein with the seven helicase domains and the causative mutations present in cell lines used in this study.
Fig. 5.2 - NER activity of XPD mutant cell lines.

A - Different amounts of XP-D WCEs were tested in dual incision assay as indicated; cell extracts were complemented either by addition of 30 µg 43-3B WCE or by 1 or 2 µl of TFIIH Hep; the amount of HeLa WCE used in lane 1 was 17.5 µg; results were quantified on a phosphorimager, where the density of bands corresponding to the 26-30 mer products was measured (units are arbitrary).

B - Different amounts of XP-D WCEs were tested in dual incision assay as indicated; cell extracts were complemented by addition of 1 or 2 µl of TFIIH Hep; the amount of HeLa WCE used in lane 1 was 17.5 µg.

C - Different amounts of XP-D WCEs were tested in dual incision assay as indicated; cell extracts were complemented by addition of 2 µl of TFIIH Hep; the amount of HeLa WCE used in lane 1 was 25 µg; the amount of XP-D WCEs used in complementation reactions (lanes 5, 8 and 11) was 50 µg.
Chapter V - XPD mutants

B

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

35  
27  

HeLa  

TFIIH  
Hep  

22 44 88 22 44

XP12BR  

XP7BE

C

1 2 3 4 5 6 7 8 9 10 11

HeLa  

TFIIH  
Hep  

25 50 68 μg 25 50 μg 25 50 μg

XP8BR  

TTD5BR  

XP1NE

135
The NER activity of these XP-D cell extracts can be correlated with the causative mutations in XPD represented in figure 5.1.

Both XP7BE and XP8BR harbour causative mutations (R683W and G675R, respectively) in the C-terminal part of XPD (Table 5.1, Fig. 5.1), a region where a significant proportion of XP group D causative mutations have been detected (Taylor et al. 1997). They are very UV-sensitive (Johnson and Squires 1992; Broughton et al. 1995) and the lack of dual incision formation is in agreement with their NER deficient phenotype (Johnson and Squires 1992). R683W and G675R are amino acid substitutions that prevent XPD interaction/stimulation with p44 therefore interfering with the helicase activity of this protein (Coin et al. 1998b).

XP12BR harbours a mutation also found in the C-terminal half of XPD. The patient from whom this cell line was established has been assigned to XP group D and is homozygous for the R511Q amino acid substitution (A. R. Lehmann and B.C. Broughton, personal communication). This amino acid change, might also prevent an association with p44. However, since this is a newly found mutation, no detailed molecular analysis has yet been done.

The XPB mutation in XP11BE cells is outside the DNA helicase domains, and results in an altered C-terminal 42 residues (Weeda et al. 1990). Although this TFIIH cannot support the 5' NER incision, the 3' incision can still take place, as observed when whole cell extracts from this patient were analysed for NER activity (Evans et al. 1997b). During this study XP11BE was used as a control (Figs. 5.2 lanes 12-15, 5.4 lanes 2-5 and 5.5 lanes 5-6).

The mutation in TTD5BR results in an amino acid substitution (A594P) in the C-terminal domain of XPD (Taylor et al. 1997). The causative mutation in XP1NE results in an amino acid substitution within the nucleotide binding motif (Walker type A) (Taylor et al. 1997). XP1NE is the only cell line tested in this part of the study where the causative mutation is in the N-terminal region of XPD (Table 5.1 and Fig. 5.1). Both TTD5BR and
XP1NE showed low levels of dual incision formation.

TTD5BR showed dual incisions at a level which is about 30% that attained by cross-complementation of XP-D cell extracts with an ERCC1 defective extract (Fig. 5.2A lanes 4, 9, 15, 19). The activity shown by XP1NE cell extract was lower. Both activities could be further stimulated by addition of purified TFIIH (Fig. 5.2C lane 8 and 11) or 43-3B WCE (Fig. 5.2A lane 4). As the causative mutation in XP1NE cells leads to an amino acid substitution within the ATP-binding motif (G47R), it was anticipated this mutation would render the helicase inactive by preventing ATP hydrolysis (Taylor et al. 1997). Additionally, a rad3 mutant allele bearing the equivalent mutation in S. cerevisiae displays extreme UV-sensitivity (Song et al. 1990). This led to an expectation of a complete NER defect in this cell line that was not observed (Fig. 5.2C lanes 9-10). In section 5.4, a detailed study of TFIIH containing an XPD subunit mutated at a different amino acid residue in this same Walker type A motif is reported and these mutations in XPD will be further discussed at the end of this chapter.

5.3 NER activity of TFIIH purified from cells with mutant XPB, XPD and p44

As previously mentioned, different mutations in the two TFIIH helicase subunits, XPB and XPD, lead to a variety of human syndromes including XP, CS and TTD (reviewed in (de Laat et al. 1999)). Moreover, it was found that the gene encoding p44 is duplicated in the chromosome 5q13 region. The more telomeric of the two genes (p44t) is located in a region associated with Spinal Muscular Atrophy (SMA) whereas the other (p44c) is more centromeric. The two p44 gene products differ by three amino acids (Bürglen et al. 1997; Tirode et al. 1999). p44t is deleted in cells from patients suffering from the most severe form of SMA (type I SMA - Werdnig-Hoffmann disease). As previously mentioned, the interaction between p44 and XPD was shown to be important for the helicase activity of TFIIH (Coin...
et al. 1998b). It was therefore clearly important to examine the NER activity of TFIIH purified from cells carrying alterations in various subunits.

In order to do this, TFIIH was immunoprecipitated using an antibody against the p44 subunit (Coin et al. 1999). In reconstituted reactions the activity of TFIIH immunopurified from the repair proficient cell lines HeLa and MRC5 (an SV40-transformed human fibroblast, for example see (Mackenney et al. 1997)) was compared with TFIIH from an XP-B cell line, an XP-D cell line, a TTD group A cell line, and two cell lines derived from patients with SMA.

As expected, TFIIH purified from HeLa and MRC5 cells was active in dual incision (Fig. 5.3 lanes 3 and 5). Also, TFIIH from the XP-B cell line GM2252 - patient XP11BE was defective in placing dual incisions around a DNA lesion (Fig. 5.5 lane 10). Whereas TFIIH purified from GM1855, a cell line derived from the asymptomatic mother of patient XP11BE (Hwang et al. 1996), was active in NER (Fig. 5.3 lane 9).

TFIIH with an XPD mutation was purified from HD2 cells and although the TFIIH supported a very low level of repair, it was not zero (Fig. 5.3 lane 6). HD2 is an immortalised cell line derived from fusion of XP102LO XP-D cells with HeLa cells, with subsequent selection of a UV sensitive clone (Johnson et al. 1985). XP102LO cells carry a R683W mutation at the C-terminal end of one allele, and an L416V substitution as well as a deletion of amino acids 716-730 in the other allele (Takayama et al. 1995). The causative mutation in HD2 is assumed to be R683W as this mutation determines the phenotype in patient XP102LO (Taylor et al. 1997). The R683W amino acid substitution is in a region of the XPD gene where many XP-D causative mutations are present (Taylor et al. 1997), and this region of the protein is involved in the interaction between XPD and p44 (Coin et al. 1998b).
Fig. 5.3 - NER activity of immunoprecipitated TFIIH containing mutations in different subunits.

TFIIH immunoprecipitated from different normal, mutated in XPD or in XPB cell lines was tested in reconstituted dual incision assay as indicated; the amounts of TFIIH used were TFIIH Hep - 1.5 μl, MRC5 TFIIH - 2 μl, TTD1BR, HeLa, HD2, Rabbouch, Djakoune, GM1855 and GM2252 TFIIH - 3 μl.
Repair in HD2 cells as measured by UDS and sensitivity to UV was reported to be slightly higher than that of the XP-D parental cell line XP102LO (Johnson et al. 1985) and it seems plausible that some residual activity might be contributed by a low level of expression of normal TFIIH in the hybrid HD2 cells.

The activities of TFIIH from cells containing only the p44c gene (Rabbouch, Fig. 5.3 lane 7) and a TFIIH containing both p44t and p44c (Djakoune, Fig. 5.3 lane 8) were compared. TFIIH activity in NER was not affected by the absence of p44t. The helicase activity of XPD in these cell lines has been shown to be normal (Coin et al. 1999) and TFIIH containing only p44c is known to be proficient in transcription (Tirode et al. 1999). These data are consistent with the absence of clinical symptoms related to NER in SMA patients (Bürglen et al. 1997).

Finally, repair in TFIIH from cells of TTD1BR, in the TTD-A group, was examined. Unlike other TTD cell lines, TTD1BR mutations in a TFIIH subunit have not been found. This is a puzzling observation as the TTD1BR repair defect can be complemented by microinjection of TFIIH complex (Stefanini et al. 1993; Vermeulen et al. 1994). The defect giving rise to TTD group A is thought to reside in a TFIIH-associated factor. Reactions containing TFIIH from TTD1BR cells formed low levels of dual incisions (Fig. 5.3 lane 4), consistent with the result observed previously with whole cell extracts from TTD1BR (Evans et al. 1997b). This indicates that TFIIH from TTD1BR cells is capable of functioning in NER, although with lower activity. The reason for the lower activity is still unknown but it could be caused, for example, by an altered posttranslational modification in TTD-A cells, such as phosphorylation.

5.4 ATP binding site (K48R) XPD mutation

To investigate the role of ATP hydrolysis by the XPD helicase subunit
of TFIIH during NER, TFIIH complexes containing wild type and mutant XPD were studied in collaboration with G. S. Winkler and G. Weeda (Erasmus University, Rotterdam). G. S. Winkler generated a wild type XPD containing a C-terminal HA-epitope tag and a similarly HA-tagged K48R mutant protein. In the mutant protein, the highly conserved lysine residue in the Walker type A nucleotide binding motif was replaced by arginine, maintaining the positive charge (GKT→GRT) (Fig. 5.1 and (Walker et al. 1982)). NER deficient CHO cells (UV5) carrying a mutation in the XPD gene (Weber et al. 1994), were transfected with cDNA encoding wild type and K48R protein and independent clones expressing either HA-tagged XPD were isolated. Highly purified wild type and K48R XPD complexes from WCEs were obtained by a method previously employed for the purification of HA-tagged XPB (Winkler et al. 1998). TFIIH complexes obtained by this approach were shown to contain all nine subunits and to have comparable concentrations (Winkler et al. 1999).

G. S. Winkler studied the enzymatic activities, transcription and repair synthesis activity of both wild type and mutant TFIIH complexes. TFIIH containing K48R mutant XPD had reduced ATPase activity (the residual activity due to a functional wt XPB ATPase), was completely deficient in XPD helicase activity (tested as ability to release labelled oligonucleotide) and was as efficient as the wild type complex in CTD phosphorylation (Winkler et al. 1999). Interestingly, TFIIH containing this inactive XPD helicase subunit was able to support a full level of transcription initiation in an in vitro reconstituted assay and showed wild type levels of formation of the transcription open complex. In a similar study, Tirode et al. produced in the recombinant form TFIIH complexes with the K48R mutation in XPD and showed that these complexes could carry out RNA synthesis but not up to wild type levels of transcription. Furthermore, a TFIIH complex containing only the 5 core subunits possessed a weak but significant transcriptional activity (Tirode et al. 1999). Together, these comparative analyses between
wild type and mutant K48R TFIIH suggest that the XPD helicase activity is dispensable for transcription initiation by RNA polymerase II in vitro.

This K48R mutation has not yet been identified in humans, but interestingly a mutation in the Walker type A domain has been described (G47R - see figure 5.1) causing an XP phenotype (Taylor et al. 1997). Additionally, biochemical analysis of the corresponding mutation in the S. cerevisiae Rad3, has demonstrated that the equivalent mutation dramatically impairs ATP hydrolysis despite the protein retaining the ability to bind ATP (Sung et al. 1988). Yeast cells carrying the same mutation are viable, but hypersensitive to killing by UV radiation, indicating that the mutation of the Lys-48 to arginine results in the inactivation of NER but not transcription (Sung et al. 1988; Sung et al. 1996). However, a cellular assay for incision using a cdc9ts (ligase) strain indicated that the rad3 Arg-48 mutant can nick DNA damaged by UV-light but it is unable to remove pyrimidine dimers from the incised DNA (Sung et al. 1988). Furthermore, low levels of nicking of closed-circular UV-irradiated DNA were also detected when Rad3 Arg-48 mutant protein was used in a reconstituted in vitro incision reaction suggesting that perhaps one incision could form (Sung et al. 1996).

For these reasons, the activity of the human TFIIH complex containing a mutant K48R XPD subunit was analysed in NER.

Using a DNA substrate containing a single 1,3-d(GpTpG) intrastrand cisplatin crosslink, the formation of dual incisions around a DNA lesion was studied in detail.

The activity of the mutant TFIIH in NER was assessed using both a fully reconstituted system (Fig. 5.4A) and by complementation of two TFIIH defective cell lines (Fig. 5.4B):

- GM2252 (patient XP11BE), bearing a mutation in XPB;
- GM2485 (patient XP7BE), bearing a mutation in XPD.
Fig. 5.4 - NER activity of purified TFIIH containing a defined mutation on the ATP-binding domain.

A - wt and K48R mutant TFIIH were tested in reconstituted dual incision assay; TFIIH Hep -1.5 μl; volume of TFIIH wt added was 1.5 or 3 μl as indicated; volume of TFIIH K48R added was 1.5 or 3 μl as indicated.

B - wt and K48R mutant TFIIH were tested in complementation of XP-B and XP-D WCEs; amount of HeLa WCE was 17.5 μg; XP-B was 22 μg and XP-D was 21 μg; the volume of TFIIH Hep added for complementation was 1 μl and wt and of K48R mutant TFIIH were 2 μl; the weak bracketed bands (lane 9) occasionally arise from non-specific end-labelling of the complementary oligonucleotide used in the detection method.
In the presence of the purified NER factors, wild type immunopurified TFIIH allows the formation of incisions on both sides of the lesion as does TFIIH purified by traditional chromatography (TFIIH Hep) (Fig. 5.4A lanes 1-4). However, reaction mixtures containing TFIIH with mutant K48R XPD subunit showed no detectable release of damaged oligonucleotide (Fig. 5.4A lanes 5-6). From other experiments it is estimated that the assay would detect dual incision activity as low as 5% of the wild type level. So, TFIIH containing K48R mutated XPD was considered inactive in dual incision formation.

Yet, mutant K48R XPD TFIIH could weakly complement an extract derived from XP-B cells (Fig. 5.4B lane 5). Correction of the incision defect by the mutant complex was lower than correction by wild type TFIIH, presumably because exchange of TFIIH subunits is needed for complementation of extract containing mutated XPB with mutant XPD TFIIH complex. Consistent with this, addition of wild type or mutant complex to extracts from XP-D cells rescued the NER activity only when the wild type was used (Fig. 5.4B lane 9). In Fig. 5.4B lane 9, two bands corresponding to DNA products of about 35 nucleotides long are detected. This is an artefact that occasionally arises from non-specific end-labelling of the complementary oligonucleotide used in the detection method (see chapter 2 figure 2.2 for details).

These results indicate that TFIIH with K48R mutant TFIIH is completely deficient in forming dual incisions around a DNA lesion, but that TFIIH with this mutation can exchange subunits with a TFIIH mutated in XPB. It is known that TFIIH can be detected in different subcomplexes, one of them being XPD-CAK (Rossignol et al. 1997). Additionally, different mutations in XPD interfere with the composition of TFIIH due to the interaction of this helicase with p44 (Coin et al. 1998b). Perhaps the weaker interaction between XPD and the rest of TFIIH allows for the interchange between the mutated XPD and the wild type one.
5.5 The 3' and 5' uncoupled incision activity of XPD mutants

Results presented so far suggest that most TFIIH complexes with mutations in XPD leading to XP features are defective in NER. The high levels of UDS shown by these cells were therefore not due to specific placement of the two NER incisions around the DNA lesion. If one assumes that the assembly of the NER machinery on a DNA lesion is affected by the XPD inactivating mutation, this could lead to some extent of defective opening during the NER reaction and to the placement of only the 5' incision. This would then provide a free 3'-OH group at which the DNA replication machinery could initiate DNA synthesis. The relatively high level of damage-dependent unscheduled DNA synthesis observed \textit{in vivo} might therefore be due to aberrant synthesis of DNA fragments different than 26-30 nucleotides (that are generated in the wild type NER reaction), after 5' uncoupled incisions are made. However, neither open complex formation nor uncoupled 3' incisions were ever detected \textit{in vitro} using whole cell extracts from XP-D cells of patients XP7BE and XP17BE (R683W amino acid substitution) (Evans \textit{et al}. 1997b).

Uncoupled 3' incisions have been reported to be made by one of the TFIIH mutants. Cells from patient XP11BE have TFIIH containing an XPB subunit which is the product of aberrant splicing (Weeda \textit{et al}. 1990; Hwang \textit{et al}. 1996). This alteration of the C-terminus of the protein does not inhibit 3' incisions but prevents formation of 5' incisions (Evans \textit{et al}. 1997b). 3' uncoupled incisions are also formed in XPF- and ERCC1-defective cell extracts (Sijbers \textit{et al}. 1996a). This suggests that XPB helicase might be in contact with or modulate the activity of the 3' nuclease ERCC1-XPF (Evans \textit{et al}. 1997b). Uncoupled 5' incisions have also been reported. Site-directed mutations inactivating the catalytic site of XPG endonuclease allow formation of 5' incisions without the concomitant 3' incision (Constantinou \textit{et al}. 1999). No naturally occurring XPG mutations of this type are known.
Would the XP-D cell extracts perform 5' uncoupled incisions analogous to the way XP11BE does the 3'? In *S. cerevisiae*, a low level of nicking of UV-irradiated DNA is observed when Rad3 Arg-48 mutant protein is tested in a reconstituted NER incision assay using UV-damaged DNA, but no excised fragments are simultaneously detected (Sung et al. 1988; Sung et al. 1996). To establish whether XPD mutant TFIIH functions in the formation of uncoupled 3' or 5' incisions, the single lesion DNA substrate was labelled at the 3' end with respect to the lesion (section 2.14.3) and incubated with cell extracts (Fig. 5.6). This assay is designed to detect all 3' incisions, arising during the dual incision reaction or as uncoupled 3' or 5' incisions (see Fig. 5.5 and diagrams on the right of Figure 5.7). All 3' incisions are detected, but 5' incisions are only detected if made in the absence of 3' incisions.

![Diagram showing 5' and 3' incisions](image)

Fig. 5.5 Scheme representing the sizes of the fragments generated by dual incision or uncoupled incisions on the *Avall* cut Pt-GTG substrate; the star represents the site of 3' $^{32}p$ labelling of the adducted strand.

Extracts from XP8BR, XP7BE, TTD5BR and XP1NE XP-D cells were compared with XP11BE, which contains the mutant XPB helicase that displays 3' uncoupled incision activity (Evans et al. 1997b) as well as XP2OS (XP-A cell line GM2345) and XP2BI (XP-G cell line XPG415A) both known to be completely defective in 3' and 5' incision formation (Evans et al. 1997a; Evans et al. 1997b).
Fig. 5.6 - Incision activity of XPD mutant cell lines.

XP-D mutant WCEs were tested for their 3' and 5' incision activity in comparison with cell extracts with known incision patterns; the amounts of each WCE were as follows HeLa - 25 μg; XP-A (XP2OS) - 25 μg; XP-B (XP11BE) - 25 μg; XPG415A - 25 μg and the various XP-D cell extracts were as indicated; lanes containing TFIIH Hep (lanes 6, 9, 12, 15 and 18) had 2 μl; XPA - 100 ng; XPG - 50 ng; all lanes except lane 1 contain aphidicolin to a final concentration of 60 μM; lane 21 contained only radiolabelled DNA substrate not subjected to any reaction; arrows indicate the positions of 3' and 5' incisions, for more detail see figures 5.5 and 5.7.
Fig. 5.7 - Incision activity of purified TFIIH containing mutated XPD helicase.

Purified TFIIH containing a defined mutation in XPD helicase subunit was tested for 3' and 5' incision activity; Lane 1 - no TFIIH; lanes 2-5 contained 1.5 µl of Hep TFIIH; lane 3 had no XPG and lanes 4 and 5 contained 0.75 ng and 1.5 ng, respectively, of E791A mutant XPG; lanes 6-12 contained the indicated TFIIH complexes: 1.5 and 3 µl of wt and K48R TFIIH, 3 µl of TFIIH immunopurified from HeLa, HD2 and TTD1BR cell extracts; lane 13 contained only radiolabelled DNA substrate; positions of incisions are as indicated.
Reactions (with the exception of lane 1) were performed in the presence of aphidicolin (inhibitor of polymerases δ and ε, that are implicated in NER (Shivji et al. 1995)) and in the absence of nucleotides. This would enable the incision events to be detected avoiding further processing by the replication machinery present in these extracts. Interestingly, aphidicolin also inhibited some 5'→3' exonuclease activity in the cell extracts. Comparison of lanes 1 and 2 shows that in the absence of aphidicolin (lane 1) there was some degradation of the labelled product that cannot be detected when this inhibitor is added (lane 2). 3’ incisions were formed by repair proficient HeLa (Fig. 5.6 lanes 1-2), and a lower level by TTD5BR (lanes 10-11) and XP1NE (lanes 13-14) extracts all of which make dual incisions (Fig. 5.2).

Additionally, 3’ incisions could be detected upon complementation of any of the cell extracts with TFIIH Hep (Fig. 5.6 lanes 9, 12, 15, 18). Uncoupled 3’ incisions were not detected except in the reactions containing XP11BE extracts (Fig. 5.6 lane 5).

No other incisions were detected; 5’ uncoupled incisions were not identified nor were any other specific incisions around the lesion or anywhere else in the plasmid substrate, with any of the cell extracts tested (Fig. 5.6).

This study was performed using whole cell extracts. Although the DNA polymerase activity had been inhibited, one cannot completely rule out the possibility that other enzyme activities in the cell extracts could mask the incisions, degrade them, or obscure their detection. To further clarify the 3’ and 5’ incision activity of XPD mutant TFIIH, purified TFIIH containing a mutant K48R XPD (section 5.3) and wt TFIIH were tested in the assay that detects 3’ and 5’ incisions, in combination with other purified NER proteins (Fig. 5.7).

In the presence of wild type TFIIH, 3’ incisions were readily formed (during the dual incision reaction - Fig. 5.2). No 5’ or 3’ incisions were
detected when the reactions were performed with TFIIH having K48R mutant XPD subunit (Fig. 5.7, lanes 6-9). As a positive control, uncoupled 5' incisions were efficiently placed in the presence of E791A mutant XPG protein as previously observed (Constantinou et al. 1999). 3' incisions were also formed by TFIIH immunopurified from HeLa, HD2 and TTD1 cells (Fig. 5.7 lanes 10-12), and these were all consistent with the dual incision activity present in these cells (Fig. 5.3). No additional 3' or 5' uncoupled incisions were detected in reaction mixtures containing XPD mutant TFIIH.

5.6 Repair synthesis activity of XP-D cell extracts

Unscheduled DNA synthesis (UDS) is defined as DNA synthesis that takes place outside S-phase in a damage dependent manner and can be a measure of repair synthesis (Rasmussen and Painter 1964). An assay using cell lysates that allows detection of specific repair synthesis of a defined adduct was used to further analyse XPD mutant cell extracts (section 2.14.5 and section 4.6). This assay permits the specific detection of repair synthesis from filling the 24-32 nt gap during NER in the presence of 32P labelled nucleotides. Cleavage of the single lesion molecule with BstNI generates a 33 nt fragment encompassing the repair site and several larger fragments flanking the lesion (Moggs et al. 1996). Specific NER synthesis should be largely localised on a 33 nt and a 68 nt fragments, labelled C and B in this figure, respectively (see section 4.6).

All five XP-D cell extracts (Table 5.1) were analysed in this assay (Fig. 5.8). There was a small amount of repair synthesis detected in XP1NE and TTD5BR (Fig. 5.8 lanes 12, 13, 18, 19), resulting from their dual incision activity. None of the other cell extracts showed any specific incorporation in any of the fragments generated by BstNI cutting. Repair synthesis was restored by addition of purified TFIIH, in all cases.

No XPD deficient cell extracts showed any repair synthesis in the
absence of complementing activity despite their high UDS levels. Lack of repair synthesis of a defined adduct means that not only there is no repair synthesis following dual incision, but also that no damage specific DNA synthesis is acting in this substrate. Non-specific DNA repair synthesis includes any synthesis event that could be started by an incision in one of the DNA strands, in a damage-dependent manner. DNA synthesis acting anywhere around the lesion would be detected by this assay even if present in the non-damaged strand.

In summary, these data indicate that the reported UDS levels in XP7BE, XP8BR and XP12BR cells do not correlate to specific NER synthesis or aberrant synthesis at uncoupled 3' or 5' incisions in vitro.
Fig. 5.8 - Repair synthesis activity of XPD mutant cell lines.

XP-D mutant WCEs were tested for their repair synthesis activity; the amount of HeLa WCE used in lane 1 was 25 μg; the amount of XP-A (XP20S) WCE was 25 μg; the amount of XP-B (XP11BE) WCE was 25 μg and the amounts of the various XP-D whole cell extracts were as indicated; lanes containing TFIIH Hep (lanes 5, 8, 11, 14, 17 nd 20) have 2 μl; XPA is 100 ng; A, B, C, D and E correspond to the BstNI fragments represented at the top of the figure.
5.7 Discussion

5.7.1 XPD mutations in NER

Most of the XP-D patients known so far carry mutations in the C-terminal half of the XPD protein. In this study I compared the NER activity with the pattern of incisions performed by TFIIH with mutations in the C-terminal domain of XPD: XP7BE, XP8BR, XP12BR, TTD5BR and HD2. Additionally, two XPD mutations in the Walker type A ATP binding motif, XP1NE and K48R, were also included (see Table 5.1 and Fig. 5.1).

Both R683W, G675R and R511Q (XP7BE, XP8BR and XP12BR, respectively) give rise to a TFIIH complex containing an XPD protein that is inactive both in dual incision formation and in the placement of any of the incisions around a lesion. R683W and G675R had already been reported to fail to interact with p44 as measured by the lack of co-immunoprecipitation of the expressed polypeptides with anti-XPD or anti-p44 antibodies (Coin et al. 1998b). The same might be happening with other mutations in this region of XPD, like XP12BR. Lack of interaction of XPD with p44 could shift the equilibrium of TFIIH complexes towards a XPD-CAK complex (Coin et al. 1999). This XPD-CAK complex has been detected in cells (Rossignol et al. 1997). If an XPD mutation could shift some of the 9 subunit TFIIH into a 5 subunit form, the TFIIH would no longer be active in NER. TTD5BR on the other hand, also displays an amino acid substitution in this C-terminal half of XPD (A594P). In contrast, this mutation did not abolish the NER activity of the TFIIH containing this mutation in XPD (Fig. 5.2). Most probably this mutation does not interfere with p44-XPD interaction, or if it does, the disruption is less severe than in XP7BE and XP8BR. Unfortunately, there are no transcription data on this mutation. It would be interesting to know if the transcription activity of TTD5BR is impaired. It has been proposed that XPD mutations leading to the clinical manifestation of XP-D cause mainly
NER defects, whereas XPD mutations leading to TTD will affect transcription (Guzder et al. 1995c; Takayama et al. 1996; Winkler and Hoeijmakers 1998). If so, it would be expected that the transcription activity of TTD5BR would be significantly more impaired than its NER activity.

The other two mutations studied are located at the conserved nucleotide binding site of XPD. This Walker type A motif contains the very conserved Gly-Lys-Thr present from bacteria to humans (Walker et al. 1982; Sung et al. 1988; Koonin 1993). XP1NE displays an amino acid substitution from Gly → Arg (G47R) in XPD. The synthetic mutation is a Lys → Arg (K48R) and allowed for a more detailed biochemical study since it could readily be purified by use of an HA-tag. Taking into account their localisation in the XPD sequence, it is very interesting that both mutations would result in such a different phenotype. Being in the nucleotide binding motif of XPD, both mutations should interfere with ATP hydrolysis and probably render the helicase inactive. However, in contrast with K48R that gave rise to a completely null phenotype in NER, XP1NE displayed a low level of dual incision formation despite the Gly to Arg amino acid substitution in the ATP binding motif. Originally called motif A by Walker and co-workers (Walker et al. 1982), ATP binding motifs are present in a variety of very different enzymes that use ATP (Saraste et al. 1990). This is a phosphate binding loop (so-called P-loop) that typically consists of a glycine rich sequence followed by a conserved lysine and a threonine or serine (GKT/S). This lysine, which in XPD is at position 48, is thought to make hydrogen bonds with the other main residues at the P-loop, but also to the β and γ phosphates of the bound NTP (Walker et al. 1982; Saraste et al. 1990). Therefore, lysine-48 is very important as a catalytic residue in this GKT motif. Regarding these structural findings an explanation of the difference in phenotype between a TFIIH containing XPD subunit with K48R and one with G47R amino acid substitution can be attempted. In K48R the amino acid substitution might impair the ATP binding site of XPD more
significantly than that of G47R. Although both the glycine and the lysine are considered invariant amino acids in this GKT ATP binding motif (Shen et al. 1994), studies in yeast suggest that K48R still binds ATP but does not hydrolyse it (Sung et al. 1988). However, the same mutation in the E. coli UvrB ATPase (K45R) resulted in failure to confer UV resistance in a UvrB deleted background (Seeley and Grossman 1989). In light of the structure of the P-loop (Saraste et al. 1990), the role of the conserved lysine residue is catalytic, whereas the role of the glycine is perhaps more structural. Overall, the difference between a G47R and a K48R amino acid substitution could be elucidated by detailed biochemical analysis on the G47R mutant.

5.7.2 NER activity versus UDS levels

After studying the behaviour of the XPD mutant cell extracts in dual incision formation, it was observed that their NER activity did not correlate with the high UDS levels reported. All the cell lines tested had high UDS levels reported in the literature (Table 5.1) despite their low or null NER activities. Therefore I tested if UDS could be attributed to NER activity using different assays. XPD mutants showed neither 3' nor 5' uncoupled incisions and no other incision at a defined position in the DNA substrate could be detected (Figs. 5.6 and 5.7). One cannot rule out the possibility that incisions at many different places (for each DNA substrate molecule) around the lesion are being made in a damage dependent manner for some unknown reason. If different incisions were occurring scattered through the damaged DNA molecule then they would be undetectable by the approaches used.

These data indicate that in some cases TFIIH containing mutated XPD subunit is inactive in NER and there seems to be no NER dependent activity that is responsible for the high UDS levels. Nevertheless, the fact that substantial damage-dependent DNA synthesis occurs, strongly suggests that there is some kind of damage-dependent DNA processing.
Could the high UDS levels present in these cells be attributed to other cellular processes?

The apparent paradox of UV-sensitivity/high levels of UDS led Paterson and co-workers (Paterson et al. 1987) to propose that the XPD mutation results in the accumulation of an intermediate in the excision repair pathway of CPDs which in normal cells is rapidly processed and difficult to detect. According to this model, in XP-D cells the inter-dimer phosphodiester bond is readily cleaved without subsequent repair and this partial cleavage of UV lesions would account for the abortive DNA synthesis and therefore the high UDS levels (Paterson et al. 1987; Johnson and Squires 1992). However, this CPD intermediate has not been characterised further (Galloway et al. 1994).

Some XP-D cells were found to lack an activity named human AP endonuclease I (Kuhnlein et al. 1978; Johnson and Squires 1992). Both human AP endonuclease I and AP endonuclease II (HAP1) are involved in the processing of base damage during base excision repair. Subsequent studies revealed that AP endonuclease I is not a true hydrolytic endonuclease, but cleaves on the 3'-side of the AP site by a β-lyase mechanism to produce a 3'-sugar terminus (Mosbaugh and Linn 1980). In some, but not all of the XP-D cells, there is an absence of this enzyme. The relevance of this to the overall NER mechanism still remains unknown, because there is no direct correlation between high UDS levels and absence of AP endonuclease I (Johnson and Squires 1992). Additionally, this activity was found to be identical to ribosomal protein S3 (Kim et al. 1995). This is intriguing but has no apparent biological relevance to repair of nuclear DNA.

In S. cerevisiae, some rad3 mutations show increased spontaneous mutagenesis as well as UV-sensitivity. A RAD3 mutant allele (rad3-1 strain carrying a E236K amino acid substitution in the DEAH helicase domain II (Yang et al. 1996)) shows enhanced activity of mismatch repair (Yang et al. 1996).
1996), (Song et al. 1990), hinting at the participation of Rad3 protein in another cellular function. It was suggested that the mutation in RAD3 conferred a mutator phenotype, increasing the rate of production of substrates that can be acted on by mismatch repair, by for instance somehow interfering with the fidelity of DNA replication (Song et al. 1990; Yang et al. 1996). On the other hand, Rad3 can be involved in inhibiting MMR and some rad3 mutations may interfere with this activity (Yang et al. 1996).

Physical interactions have been found between Msh2 and Rad3 as well as other NER factors (Bertrand et al. 1998). Moreover, a defined mutation at the C-terminus of yeast Rad3 (rad3 - G595R) led to higher levels of short-sequence recombination implicating Rad3 in the maintenance of genome stability in S. cerevisiae (Bailis et al. 1995). Rad3 might be involved along with Rad1 and Srs2 in the removal of non-homologous DNA ends during DSB repair (Paques and Haber 1997). If this is the case, and XPD could have a similar role to Rad3 in human cells, then the high UDS-levels could be due to an increase in mismatch repair or in recombination activity. There is also evidence that some NER factors are involved in short-patch mismatch repair in yeast, showing some overlap between both repair processes, and this deserves further investigation (Fleck et al. 1999).

In order to test for this hypothesis, preliminary experiments could involve the determination of UDS at the different stages of the cell cycle.

Knowing that TFIIH containing a mutant XPD is still able to produce the open DNA structure required for transcription to start (Sung et al. 1988; Feaver et al. 1993; Winkler et al. 1999) there might be occasional structure-specific endonucleolytic cleavage of the DNA in transcription bubbles. Such nicking could provide DNA polymerases with the substrate to synthesise spurious tracts of DNA. Of course, in order to be damage-specific nicking this would mean that the transcription machinery would produce an open structure that is specific for damaged DNA. Perhaps TATA-binding protein (TBP) with its affinity for DNA lesions could falsely initiate transcription at
5.9 Role of XPD in NER

Both XPB and XPD helicases are subunits of transcription factor TFIIH. A mutation that destroys the transcriptional activity of TFIIH will be inviable. One could therefore expect that patients with mutations in XPB or XPD would be rare. But whilst XP-B are rare, many different mutations have now been identified in the XPD gene, implying that TFIIH transcriptional activity is more tolerant towards amino acid changes in XPD than in XPB. In S. cerevisiae, whereas a mutation (K48R) in Walker motif A of Rad3 produces a TFIIH that is active in transcription (Feaver et al. 1993) but not in repair (Sung et al. 1988), the same type of mutation (K392R) in the ATP binding motif of Rad25 is lethal (Park et al. 1992). Thus ATP hydrolysis in Rad25 is more important for viability than in Rad3.

Human TFIIH containing mutant XPD is unable to efficiently place both incisions around a DNA lesion (this chapter), indicating that XPD activity is required for efficient NER and UV-resistance. The same activity is not needed for transcription as shown by the viability of yeast cells bearing the same mutation (Sung et al. 1988; Feaver et al. 1993). Detailed biochemical analysis of TFIIH K48R mutant in vitro has also shown that it is active in transcription (Coin et al. 1999; Winkler et al. 1999).

One obvious difference between the biochemistry of transcription and of NER is that the TFIIH unwound region in NER is about 25-30 nt whereas in transcription it is 10-15 nt (Holstege et al. 1996; Evans et al. 1997a; Holstege et al. 1997). Perhaps the NER reaction uses the additional helicase activity in order to produce a larger open structure.

One model consistent with all these data is that XPB is the helicase essentially devoted to transcription whereas XPD, or the concerted action of both XPB and XPD, is involved in NER.
CHAPTER VI
Nucleotide excision repair protein complexes

6.1 Introduction

As described in chapter 4 the minimal set of proteins involved in the first stage of the nucleotide excision repair reaction are heterotrimeric RPA, XPA, TFIIH complex, XPC-hHR23B, XPG, and ERCC1-XPF.

Many protein-protein interactions between NER components have been found in yeast and mammalian cells (see chapter 1). An array of different interactions between the factors involved in the first steps of NER has been detected by methods that range from immunoprecipitation and affinity chromatography to yeast two hybrid systems. Most of these studies were concerned only with individual interactions between the proteins involved in NER and used detection methods that would only assess physical interactions and not functional relevance. Higher order complexes between NER proteins have also been reported and designated "repairosomes" (Svejstrup et al. 1995; He and Ingles 1997; Rodriguez et al. 1998). These complexes have been studied using different techniques and their compositions vary according both to the isolation and the detection method. A systematic comparison of relative strengths of interactions had not yet been made. Measurements of the functionality of the different protein complexes were also lacking.

In this part of my study I used immunoprecipitation techniques in order to look for the existence of any higher order complexes and examine the relative strengths of interactions between the core NER proteins. To elucidate which are the functionally relevant interactions between factors I analysed active proteins present in an NER complex by directly testing for their activity in vitro.
Fig. 6.1 - TFIIH complex is quantitatively immunoprecipitated from HeLa cell extracts with a cdk7 monoclonal antibody.

TFIIH was immunoprecipitated from a HeLa whole cell extract as described in chapter II. Detection was performed by using a monoclonal antibody against p62 subunit of TFIIH (3C9). Lane 1 contains 5 μl of Hep TFIIH; lanes 2 and 3 contain 100 μg and 50 μg of HeLa WCE, respectively; lanes 4-9 contain 20 μl of W and S fractions and 5 μl of B fraction; W - 1st wash, S - HeLa supernatant, B - beads. Control antibody is mouse whole IgG molecule. Bands were quantified using the NIH image programme.
6.2 TFIIH immunoprecipitation from HeLa cell extracts

Complexes of human NER proteins were isolated from cell extracts by immunoprecipitation. Since TFIIH has been reported to play a central role in the NER reaction (Evans et al. 1997b) and to interact with several other factors involved in the incision/excision of damage, namely XPA (Park et al. 1995; Nocentini et al. 1997), XPC (Drapkin et al. 1994) and XPG (Mu et al. 1995; Iyer et al. 1996), an antibody against the cdk7 (MO1.1) subunit of TFIIH was chosen. The cdk7 kinase is part of the CAK subcomplex of TFIIH and an antibody against this subunit has been shown to be able to immunoprecipitate transcriptionally active TFIIH from HeLa cells (Ossipow et al. 1995).

There are several advantages to using this antibody. First, cdk7 being part of CAK is a TFIIH subunit that is not needed for NER as demonstrated in chapter 4 and this lessens the chance that the antibody would sterically inhibit repair. Second, anti-cdk7 antibody was shown to immunoprecipitate transcriptionally active TFIIH (Ossipow et al. 1995) and it is able to immunoprecipitate NER active TFIIH as is shown in section 6.2.2 of the present chapter. Third, immunoprecipitated TFIIH can be used in activity assays while immobilised on beads, so no elution step is required.

6.2.1 Presence of other NER factors

TFIIH in HeLa cell extracts was immunoprecipitated by anti-cdk7 antibodies bound to magnetic beads and the input (HeLa cell extract), the supernatant (HeLa cell extract after immunoprecipitation) and beads (TFIIH bound fraction) analysed by immunoblotting the p62 subunit of TFIIH. As a control, mouse whole IgG molecule was used. TFIIH complex can be isolated in a specific manner from HeLa cells (Fig. 6.1, compare lanes 6 and 9). Under the conditions used (50 mM KCl and 0.01% Triton X-100), nearly all TFIIH
present in HeLa cells could be immunoprecipitated (Fig. 6.1 Lane 9). In contrast, in the absence of anti-cdk7 antibody, TFIIH was detected in the supernatant but not in the immunoprecipitated fraction (Fig. 6.1 lanes 5 and 6).

Having quantitatively immunoprecipitated TFIIH from HeLa cells under mild conditions (50 mM KCl and 0.01% of the non-ionic detergent Triton X-100) I therefore asked whether this TFIIH complex was interacting with other NER factors. The presence of other NER factors in the immunoprecipitate was assayed by immunoblotting (Fig. 6.2). Consistent with previous reports (Drapkin et al. 1994; Park et al. 1995; Iyer et al. 1996; Nocentini et al. 1997), I found some XPA, XPG and XPC to immunoprecipitate with TFIIH from HeLa cell extracts. Additionally, some ERCC1-XPF and hHR23B were also detected.

<table>
<thead>
<tr>
<th>repair factor</th>
<th>relative amount IP from HeLa</th>
<th>fmol IP per μl of immunoprecipitate</th>
<th>fmol added per reconstituted reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 μl</td>
<td>3 μl</td>
</tr>
<tr>
<td>TFIIH</td>
<td>100%</td>
<td>270</td>
<td>810</td>
</tr>
<tr>
<td>RPA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XPA</td>
<td>4%</td>
<td>15</td>
<td>45</td>
</tr>
<tr>
<td>ERCC1</td>
<td>4%</td>
<td>15</td>
<td>45</td>
</tr>
<tr>
<td>XPF</td>
<td>4%</td>
<td>15</td>
<td>45</td>
</tr>
<tr>
<td>hHR23B</td>
<td>1%</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>XPC</td>
<td>36%</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>XPG</td>
<td>15%</td>
<td>35</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 6.1 - Amounts of repair proteins present in the TFIIH bound fraction, in relation to the total amount detected in HeLa cell extracts. Quantification of the amounts of proteins present in the immunoprecipitate in fmol (column 3) was performed by Western blot in comparison with known quantities of recombinant protein. These values are result of various extrapolations and are not absolute, they serve only as a basis for comparison (for details of the quantification see Appendix 3). Values presented in column 4 are calculated from the amounts of recombinant protein added per NER reaction.
Fig. 6.2 - Co-immunoprecipitation of TFIIH and other NER factors.
TFIIH was immunoprecipitated from a HeLa whole cell extract as described in Materials and Methods. Detection was performed by using various a antibodies against the different proteins involved in the first steps of NER, as specified on the left hand side. Lanes 1 and 2 contain (from top to bottom) 2.5 and 5 μl TFIIH Hep, 125 and 250 ng RPA, 22.5 and 45 ng XPA, 75 and 150 ng XPC-hHR23B, 50 and 100 ng ERCC1-XPF and 125 and 250 ng XPG; lanes 3-5 contain 17.5 μg, 35 μg and 70 μg of HeLa WCE, respectively; lanes 6-9 contain 17.5 μg, 35 μg and 70 μg of the same extract after immunoprecipitation (Sup); lane 10 contains 20 μl of beads.
In quantitative immunoblotting experiments (Fig. 6.2) I estimated the total amount of protein that was being immunoprecipitated from the HeLa cell extracts. Assuming that the amount of TFIIH immunoprecipitated from HeLa cells is 100%, the quantity of this complex being pulled down with the beads, can be compared with the relative amounts of the other proteins detected. In Table 6.1 a comparison of the estimated relative amounts of NER proteins (in relation to the total amount in the HeLa cell extract) immunoprecipitated with the anti-cdk7 antibody is shown. About 36% of the total XPC and 15% of the total XPG present in HeLa cell extracts was brought down in the TFIIH fraction. However, the amounts of other proteins present in this fraction are below 5% of the total amount detected in HeLa cells. Using these values it is also possible to calculate the amount of TFIIH complexed with XPC in cells. The percentage of TFIIH complex that is interacting with XPC is about 8%. There is an excess of TFIIH in comparison with XPC and ≈ 8% could be part of a subcomplex for NER.

According to van der Spek et al. ≈ 2-4 x 10⁵ molecules of hHR23B and ≈ 4-8 x 10⁴ of XPC are present in a single HeLa cell (van der Spek et al. 1996). Applying these figures to the data originated by the quantitation of the amounts of each protein present in the immunoprecipitates, it is clear that approximately the same values are determined (see Appendix 3). 36% of the total of XPC molecules present in a single HeLa cell was immunoprecipitated with TFIIH and this corresponds to 17.5 - 35 fmol of XPC. hHR23B is present as 1% of the total and this value corresponds to 5-10 fmol. The values I calculated are in accordance with the quantification reported by van der Spek et al. for XPC-hHR23B in relation to the total amount of XPC and hHR23B in HeLa cells.

Taken together these results indicate that under these conditions XPA, XPC-hHR23B, ERCC1-XPF, XPG and TFIIH could be detected together in a HeLa cell immunoprecipitate, and that a proportion of XPC (36%) and XPG (15%) proteins present in the cell is interacting with TFIIH.
Fig. 6.3 - TFIIH interacts with different NER factors in a functional complex.

Cdk7 beads were used in dual incision assay as described. 3 µl magnetic beads containing TFIIH and associated factors were added to a reconstituted dual incision assay and protein-protein interactions tested by sequential omission of each repair factor as indicated. Lanes marked "Complete" contain all the repair factors; TFIIH interactions were studied at 50 mM KCl concentration; lane 2 contains 1.5 µl Hep TFIIH; lanes 3 and 4 (Complete (1) and (2)) contain 1.5 µl and 3 µl of cdk7 magnetic beads, respectively; lanes 5-9 contain 3 µl of beads; quantification was performed on a phosphorimagerr relatively to the lane “complete” containing 3 µl TFIIH beads (lane 4).
6.2.2 Functional NER interactions

Since Ossipow et al. immunoprecipitated a TFIIH complex that is active in transcription (Ossipow et al. 1995), the NER activity of the previously described immunoprecipitates was tested. Interestingly, I found that the TFIIH bound to the anti-cdk7 antibody-bead complex was active in NER \textit{in vitro}. The assay used (dual incision assay) detects the dual incisions characteristic of NER by directly labelling the damaged excised fragment (Fig. 6.3 lanes 3 and 4). The immunoprecipitated TFIIH has comparable activity to TFIIH purified from HeLa cells over 4 chromatographic steps (TFIIH Hep) (Fig. 6.3 compare lanes 2 and 3). Functionality of interactions between the individual factors and TFIIH complex was tested by omitting each purified factor from the reactions containing the immunoprecipitates. For example, if NER activity is detected in the absence of recombinant XPC-hHR23B complex, then it is due to the presence of XPC-hHR23B in the TFIIH bound fraction. Reactions containing either XPC or hHR23B alone do not show any significant dual incision activity (D. Batty, unpublished).

The only case in which no dual incision products were detected was when RPA was omitted. In the absence of either XPA, XPC-hHR23B or XPG the dual incision activity is only reduced to about 20-25% (Fig. 6.3 compare lane 4 to lanes 6-9) and when ERCC1-XPF is omitted from the reaction, NER activity is reduced to approximately 10%. This indicates that some XPA, XPC-hHR23B, XPG and ERCC1-XPF are present in the TFIIH bound fraction and are functional in NER when immunoprecipitations are performed at 50 mM KCl and 0.01% Triton X-100.

It is not surprising that the NER activity of the reactions in the absence of XPA, XPC-hHR23B, XPG and ERCC1-XPF was below the activity of the fully reconstituted reaction. Reactions containing immunoprecipitates and pure repair factors have an excess of NER enzymes in comparison to reactions where one of each of these factors has been omitted (Table 6.1).
To confirm the functionality of the interactions between the NER factors the NER activity of the immunoprecipitates alone and upon addition of RPA was tested (Fig. 6.4). The immunoprecipitate is active in dual incision formation when recombinant RPA complex is added to the reactions. The NER activity of reactions containing RPA plus the TFIIH bound immunoprecipitate was about 20% of the activity shown by addition of the same immunoprecipitate to the full set of NER proteins (Fig. 6.4 compare lane 3 to lanes 4 and 5).

The amounts of NER proteins present in the TFIIH bound fraction can be roughly estimated by quantitative immunoblotting (Fig. 6.2 and Table 6.1). In these reactions 10 μl of the immunoprecipitated beads were added and two different amounts of recombinant RPA were tested. Lanes containing only the immunoprecipitates and recombinant RPA (Fig. 6.4 lanes 4 and 5) had total NER proteins with a concentration within an order of magnitude as the concentrations in the fully reconstituted reaction (Fig. 6.4 lane 1). Exceptions are RPA and TFIIH, which were in excess in the reactions that just contained recombinant RPA and the immunoprecipitate. The NER activity of the TFIIH bound fraction after addition of RPA is comparable to the activity of a reaction containing individually added recombinant RPA, XPA, XPC-hHR23B, XPG and ERCC1-XPF and human purified TFIIH (Fig. 6.4 compare lane 1 with lanes 4 and 5). In this fully reconstituted repair reaction (where each recombinant factor was added individually), the amounts of each of the factors are as follows: RPA 450 fmol, XPA 700 fmol, XPC-hHR23B 80 fmol, XPG 340 fmol, ERCC1-XPF 60 fmol and 500 fmol of TFIIH Hep fraction (see Table 6.1). Whereas in the reaction in lane 4, the amounts of each protein (since 10 μl of beads were used) were estimated as 150 fmol XPA, 100 fmol XPC-hHR23B, 50 fmol ERCC1-XPF and 340 fmol XPG, plus 1.1 pmol RPA and 2.7 pmol TFIIH (1 μl of beads is equivalent to 1 μl of TFIIH Hep fraction).
Fig. 6.4 - Adding RPA to the HeLa immunoprecipitates restores their NER activity

10 µl of magnetic beads containing TFIH immunoprecipitated (TFIIH IP) at 50 mM KCl and associated factors were added to a reconstituted dual incision assay either in the presence of all repair factors (lane 3), only in the presence of RPA (lanes 4-5) or alone (lane 6); lanes 4 and 5 contain 450 fmol and 1100 fmol RPA, respectively; complete (lanes 1-3) contain all recombinant repair factors and no TFIH, TFIH Hep or IP TFIH as indicated; lanes marked IP TFIH (4-6) contain only 10 µl of magnetic beads and RPA where indicated.
Fig. 6.5 - Protein interactions between XPG and other NER factors.
XPG was immunoprecipitated from HeLa extracts by anti-XPG (8H7) monoclonal antibody and XPG beads were used in dual incision assay. 3 μl magnetic beads containing XPG and associated factors were added to a reconstituted dual incision assay and protein-protein interactions tested by sequential omission of each repair factor as indicated. Lanes marked "Complete" contain all recombinant repair factors, HeLa purified TFIIH and immunoprecipitated XPG or recombinant XPG; lane 1 contains 45 ng baculovirus recombinant purified XPG protein; lanes 3-8 contain 3 μl of XPG magnetic beads.
Chapter VI - NER complexes

These results indicate that in immunoprecipitations done at 50 mM KCl, 0.01% Triton X-100 the amounts of proteins in 10 μl of TFIIH bound fraction are sufficient to perform in vitro repair to the level of a mixture of pure recombinant factors and HeLa purified TFIIH (Fig. 6.4 lane 1 and 4-5). With the exception of TFIIH, which is in excess and of RPA which is absent from the immunoprecipitate, the other incision factors are present in amounts that are the same order of magnitude to the amounts added to a reconstituted reaction (Table 6.1 and Appendix 3).

6.3 XPG immunoprecipitated from HeLa cell extracts

As an independent test of whether the repair proteins present in the TFIIH immunoprecipitate were interacting with each other, the same experiment was performed using an antibody against XPG protein. Using the same experimental approach the activity of XPA, XPC-hHR23B, ERCC1-XPF and TFIIH was detected in association with XPG in HeLa cell extracts (Fig. 6.5 lanes 5-8). As with the immunoprecipitates done with anti-cdk7 antibody, no functional RPA was immunoprecipitated (Fig. 6.5 lane 4). So, XPA, XPC-hHR23B, ERCC1-XPF, XPG and TFIIH complex are present and functional in a fraction immunoprecipitated from HeLa cells using an antibody that recognises XPG.

6.4 Strong interaction between TFIIH and XPC-hHR23B

The results presented above suggest the existence of a higher order complex between the proteins involved in the first stages of NER. However the conditions used for this isolation are relatively mild.
Fig. 6.6- TFIIH interacts with XPC and XPG in a functional complex at 150 mM KCl.
Cdk7 beads were used in dual incision assay as described. 3 µl magnetic beads containing
TFIIH and associated factors were added to a reconstituted dual incision assay and
protein-protein interactions tested by sequential omission of each repair factor as
indicated. Lanes marked "Complete" contain all the recombinant repair factors and no
TFIIH, TFIIH Hep or immunoprecipitated TFIIH bound to cdk7 beads; TFIIH interactions
were tested at 150 mM KCl, 0.01% Triton-X100; lane 2 contains 1.5 µl Hep TFIIH; lanes 3-9
contain 3 µl of beads; quantification was performed on a phosphorimager relatively to the
lane "complete" containing 3 µl TFIIH beads (lane 3).
Fig. 6.7- TFIIH interacts strongly with XPC-hHR23B.

3 μl magnetic beads containing TFIIH and associated factors were added to a reconstituted dual incision assay and protein-protein interactions tested by sequential omission of each repair factor as indicated. Lanes marked "Complete" contain all recombinant repair factors and immunoprecipitated TFIIH; TFIIH interactions were studied at 300 mM and 500 mM KCl concentrations; lane 2 contains 1.5 μl Hep TFIIH; lanes 3 and 4 contain 1.5 μl and 3 μl of cdk7 magnetic beads washed at 300 mM KCl, respectively; lanes 5-9 contain 3 μl of beads; lanes 10 and 11 contain 1.5 μl and 3 μl of cdk7 magnetic beads washed at 500 mM KCl, respectively; lanes 12-16 contain 3 μl of beads.
In order to distinguish between stronger and weaker interactions between these factors I performed the same experiments under higher stringency conditions which would disfavour weak ionic interactions (washing the beads in 150 mM KCl). Again, the immunoprecipitated TFIIH is as active as the complex purified from HeLa cells (Fig. 6.6 lanes 2 and 3). However, when each of the other NER factors is sequentially omitted, NER activity is only detected in the absence of XPC-hHR23B complex and XPG (Fig. 6.6 lanes 6 and 7). By washing the beads in more physiological salt concentrations I was able to separate a weaker interaction between TFIIH and XPA and TFIIH and ERCC1-XPF from stronger functional interactions between TFIIH and XPC-hHR23B and TFIIH and XPG. If the salt concentration was further increased to 300 mM KCl the only interaction which was functionally detectable was the TFIIH-XPC complex interaction and this one was finally disrupted at 500 mM KCl (Fig. 6.7).

6.5 TFIIH immunoprecipitated from lymphoblastoid cells

The isolation of NER subcomplexes from HeLa cells was presented so far. The same approach can be used in order to study complexes from lymphoblastoid cells. This is important to establish as it allows further studies with NER-defective lymphoblastoid cell lines which lack specific XP proteins. TFIIH was immunoprecipitated from a lymphoblastoid cell extract proficient in NER (705 ori) (Rapic-Otrin et al. 1998). The study of functional interactions with complexes precipitated from these cells is presented in Figure 6.8. As in the previously described experiments, I was able to immunoprecipitate TFIIH from this cell extract in a form that is as active as HeLa purified TFIIH (Fig. 6.8A lanes 2-4).
Fig. 6.8 - Functional interactions of the TFIIH complex in normal lymphoblastoid cells
CdK7 beads were used in dual incision assay after incubation with a 705ori WCE. 3 µl magnetic beads containing TFIIH and associated factors were added to a reconstituted dual incision assay and protein-protein interactions tested by sequential omission of each repair factor as indicated. Lanes marked "Complete" contain all the repair factors; lane 2 contains 1.5 µl Hep TFIIH; lanes 3 and 4 contain 1.5 µl and 3 µl of cdk7 magnetic beads, respectively; lanes 5-9 contain 3 µl of beads.
A - TFIIH interactions at 50 mM KCl concentration
B - TFIIH interactions at 250 mM KCl.
Quantification was performed in a phosphorimager and results plotted in relation to the "complete" reaction containing 3 µl of beads (lane 4).
Chapter VI - NER complexes

B

![Graph and gel image showing relative phosphorimager counts for different conditions.](image)

- TFIIH
- Complete
- RPA
- XPA
- XPC-hHR23B
- XPF
- ERCC1-XPF

Hep TFIIH
immunoprecipitated TFIIH
(250 mM KCl)
Analysing the functional interactions by sequential omission of each of the repair factors suggested that the complexes immunoprecipitated were similar to the ones isolated from HeLa cell extracts under the same conditions (50 mM KCl, 0.01% Triton X-100). TFIIH from lymphoblastoid cells functionally co-immunoprecipitates with XPA, XPC-hHR23B, XPG and ERCC1-XPF. When TFIIH was immunoprecipitated at higher salt concentrations (250 mM KCl, 0.01% Triton X-100) again only XPC-hHR23B was detected, as was the case with the complex from HeLa cell extracts (Fig. 6.8B).

6.6 TFIIH immunoprecipitated from XP-A defective cells

XPA binds damaged DNA and plays a central role in NER interacting with many core repair factors (reviewed in (Wood 1999) and chapter I). Having analysed the complexes immunoprecipitated from lymphoblastoid cells I set out to study the influence of XPA protein in the formation of these complexes. Extracts were prepared from a lymphoblastoid cell lacking XPA protein. GM2345 is a lymphoblastoid cell line derived from patient XP20S. This cell line is completely defective in NER, showing low levels of reduced size XPA mRNA (Satokata et al. 1990) and no detectable XPA protein ((Miura et al. 1991) and D. Batty, unpublished observations). TFIIH was immunoprecipitated from GM2345 lymphoblastoid cell extract as previously. Although this complex was isolated from XPA deficient cells, its activity in NER was similar to the activity of an HeLa purified TFIIH (Fig. 6.9 lanes 2-4) and comparable to a TFIIH immunopurified from normal lymphoblastoid cells (Fig. 6.9 lanes 3-4). However when the complexes co-immunoprecipitated with this factor were further analysed, the difference due to the lack of XPA protein in this extract could be detected. As with immunoprecipitates from normal lymphoblastoid cells an interaction between TFIIH and XPC-hHR23B, XPG and a very small quantity of ERCC1-
XPF complex was detected (Fig. 6.9 lanes 7, 8 and 9). However, as expected, no XPA protein was present in this immunoprecipitate (Fig. 6.9 lane 6).

To directly determine whether the XPA level influenced the interactions between TFIIH and the other incision factors I immunoprecipitated TFIIH from XP-A cells that had been supplemented with pure recombinant XPA protein (to the same ratio protein/cell-extract as in complementation for NER activity (Köberle et al. 1999)). XPA protein was added to a GM2345 whole cell extract and incubated for 30 min at 30°C. Following this incubation, immunoprecipitations were performed with anti-cdk7 antibody and beads washed at 50 mM KCl, 0.01% Triton X-100. Some of the protein-protein interactions observed this way are increased compared to the ones in the absence of XPA protein. First, I observed co-immunoprecipitation of XPA with TFIIH meaning that the recombinant XPA protein added as a complementing factor is now interacting with TFIIH complex. Second, XPC complex and XPG were detected as previously, but the amounts of ERCC1-XPF complex were slightly increased. Comparison of the complexes isolated before and after XPA complementation suggests that addition of XPA increases the interaction between the TFIIH, XPA and ERCC1-XPF. Additionally it shows that recombinant XPA can interact with TFIIH complex when added to a lymphoblastoid cell extract with no detectable levels of XPA protein and so in order for complexes to be detected by this technique, they do not necessarily have to be pre-assembled in cells.
Fig. 6.9 - Protein-protein interactions in XP-A and XPA complemented XP-A cell extracts.

Cdk7 beads were used in dual incision assay after incubation with an XP-A WCE. 3 μl magnetic beads containing TFIIF and associated factors were added to a reconstituted dual incision assay and protein-protein interactions tested by sequential omission of each repair factor as indicated. Lanes marked "Complete" contain all the recombinant repair factors and immunoprecipitated TFIIF. Lanes 3-9 contain reactions with TFIIF immunoprecipitated from an XP-A cell line; lanes 10-16 contain reactions with TFIIF immunoprecipitated from an XP-A cell line after addition of purified XPA protein. Lane 2 contains 1.5 μl Hep TFIIF; lanes 3, 4 and 10, 11 (Complete (1) and (2)) contain 1.5 μl and 3 μl of cdk7 magnetic beads, respectively; lanes 5-9 and 12-16 contain 3 μl of beads; quantification was performed on a phosphorimager relatively to the lane “complete” that contains 3 μl IPTFIIF beads (lane 4 to the first set of values and lane 11 for the second set).
6.7 Discussion

For the first time, I used an approach that allows the isolation of higher order complexes from mammalian cells followed by testing for their activity in an assay that detects the dual incisions characteristic of NER. Using this assay I was able to test for functional interactions between NER factors involved in the first steps of the reaction.

Immunoprecipitation of TFIIH from HeLa cells with an anti-cdk7 antibody yielded TFIIH complex-bound factors involved in dual incision formation. Most interestingly, this immunoprecipitation succeeded in isolating approximately 100% of all the TFIIH complexes in the HeLa whole cell extract. This observation indicates that most of the TFIIH present in the cell extract is actually complexed with the CAK heterotrimer, in a 9 subunit complex, in contrast with what was indicated by previous studies where distinct complexes (TFIIH, XPD-CAK and CAK) have been reported (Adamczewski et al. 1996; Drapkin et al. 1996; Yankulov and Bentley 1997). These partial TFIIH complexes are probably generated by the purification process itself. Under high salt concentration TFIIH can dissociate into several subcomplexes: CAK, CAK-XPD, core TFIIH and core TFIIH-XPD (Schaeffer et al. 1994; Rossignol et al. 1997). Apparently, 9 subunit TFIIH dissociates into several forms during the various column purification steps. According to the studies presented in this thesis, using mild extraction conditions, most TFIIH is present in cells as a 9 subunit complex. Additionally, this TFIIH complex is active in NER although it has been isolated via its interaction with CAK. Complexes immunoprecipitated in the same way have been shown to be also active in transcription (Ossipow et al. 1995). This differs from observations in Saccharomyces cerevisiae where a nine-subunit TFIIH is required for transcription (as in human cells) but a six-subunit core-TFIIH devoid of kinase components is mainly associated with repair proteins to form the repairosome that participates in NER.
In this study, I could not detect any significant amount of 6 subunit TFIIH in whole cell extracts (Fig. 6.1).

The results presented in this chapter suggest that a fraction of the proteins required for the dual incision step of NER functionally interact in vitro under mild salt concentrations. This fraction is very significant when XPC and XPG are concerned. About 36% of all the XPC and 15% of all the XPG present in a HeLa whole cell extract is complexed with TFIIH at 50 mM KCl. For other NER factors, only fractions smaller than 10% are detected in the TFIIH bound fraction. However, when the TFIIH bound fraction is supplemented with recombinant RPA and used in a dual incision assay, its activity in this assay is similar to the activity of the same amounts of protein added together to perform the same reaction. Thus a functional complex can be formed at 50 mM KCl.

If the salt concentration is increased to an ionic strength closer to the physiological level, like 150 mM KCl, the only interactions that are functionally detectable are between TFIIH and XPC-hHR23B and TFIIH and XPG (Fig. 6.6). Additionally, complexes immunoprecipitated from a cell line that does not contain detectable levels of XPA protein, show presence of only XPC-hHR23B and XPG and low levels of ERCC1-XPF (Fig. 6.9). Upon addition of XPA protein, however, TFIIH is immunoprecipitated not only with XPC-hHR23B and XPG but also with XPA and slightly higher levels of ERCC1-XPF.

Taken together these results indicate that the strongest interactions amongst NER factors are between TFIIH and XPC-hHR23B and TFIIH and XPG, with the TFIIH-XPC complex interaction being the strongest.

A schematic drawing of the interactions between TFIIH and other incision/excision factors, is in figure 6.10 where the stronger and weaker interactions have been colour coded. The strongest interaction is between XPC-hHR23B and TFIIH, followed by the interaction of XPG with TFIIH complex.
Fig. 6.10 - Schematic representation of protein-protein interactions of human NER factors.
Model of the interactions studied in human cell extracts; darker tone represents stronger interaction and lighter tone, weaker interaction as detected by functional assays.
A complex of TFIIH-XPC-hHR23B-XPG can be detected both at 50 mM and at 150 mM KCl (Fig. 6.5 and 6.6). Based on evidence coming from mobility shift assays it was proposed that TFIIH interacts with either XPC or XPG and never with both (Wakasugi and Sancar 1998). Assuming that XPC and XPG do not interact directly, the data presented here are not consistent with this model since TFIIH can be complexed with both XPC and XPG in cell extracts (Figs. 6.5 and 6.6). The assumption that XPC-hHR23B and XPG do not directly interact is most probably correct. First, it is known that XPG interacts directly with TFIIH subunits (Iyer et al. 1996). Second, from this study we know that XPC-hHR23B is found in a complex with TFIIH in the absence of XPG (Fig. 6.6). The most direct way of testing for this direct interaction between XPC-hHR23B and XPG would be by doing the same XPG immunoprecipitation from TFIIHΔ cells, which is impossible.

On the level of the weaker interactions, we have XPA and ERCC1-XPF. XPA is well known to interact with ERCC1 (Li et al. 1994; Park and Sancar 1994; Li et al. 1995b; Saijo et al. 1996; Bessho et al. 1997; He and Ingles 1997) and with TFIIH complex (Park et al. 1995; Nocentini et al. 1997). Also, when attempts were made to isolate higher order complexes from XPA defective cells we could detect hardly any amounts of ERCC1-XPF in the absence of XPA protein, whereas when this factor was added to the same extract the amounts of ERCC1-XPF complex increased simultaneously. Based on all these data, and also on the fact that ERCC1-XPF was never found to interact with TFIIH in mammalian cells, these weaker interactions were represented as XPF-ERCC1-XPA-TFIIH where TFIIH complex does not contact ERCC1-XPF. RPA is known to interact with XPA (He et al. 1995; Lee et al. 1995; Li et al. 1995a; Matsuda et al. 1995; Saijo et al. 1996; Stigger et al. 1998), however two of its subunits were not detected by immunoblot in the TFIIH bound fraction. Additionally TFIIH bound fractions at 50 mM KCl, were only active upon the addition of heterotrimeric recombinant RPA. Consequently, RPA is represented outside this "interactions complex", since no physical or
functional association was detected. The XPA-RPA interaction domains have been studied and it is thought that this interaction is very important for NER (Li et al. 1995a). Perhaps the RPA-XPA interaction is only strongly formed in the presence of DNA (Wakasugi and Sancar 1998; Wakasugi and Sancar 1999) or only XPA protein that is not interacting with TFIIH is able to interact with RPA. Matsuda et al. were able to immunoprecipitate RPA and XPA from HeLa cell extracts using an antibody against XPA (Matsuda et al. 1995), but maybe this was a very small amount undetectable in the present study.

According to this study, in the immunoprecipitate there is an excess of TFIIH but the amounts of other factors present are as active as the separately added recombinant proteins. However, what is referred to as immunoprecipitate is most probably a mixture of different complexes - TFIIH alone and different combinations of factors. Nevertheless, the proportions of different factors present are approximately in the same ratio as the proportions used for fully reconstituted NER in vitro. It is therefore conceivable that we have a percentage of fully assembled complexes existing in vivo although these would not represent more than 10% of the total repair factors in mammalian cells (Table 6.1). In a recent quantitative study of RNA polymerase II holoenzyme complexes it was found that only about 3% of the total soluble Pol II was detected as a holoenzyme size complex containing all the other transcription factors (Kimura et al. 1999). Perhaps a similar behaviour can be expected from nucleotide excision repairosomes.

Do these results reveal the most important interactions between TFIIH and other NER factors? Previous studies have shown that for a complete open DNA pre-incision structure to occur, the presence of XPA, RPA, XPC-hHR23B and XPG is necessary along with the action of TFIIH and its two helicases XPB and XPD ((Evans et al. 1997b) and D. Gunz, unpublished results). One possible NER model is that TFIIH helicases initially catalyse limited opening on the 3' of the lesion and this ssDNA area of about 10
Chapter VI - NER complexes

nucleotides is then bound by RPA. This "primary open structure" is then substrate for the binding, stabilisation and further opening which is performed by all the other factors acting in a concerted manner (Evans et al. 1997b; de Laat et al. 1998b)

Based on the present results, the strong functional interaction of XPC complex with TFIIH could mean that these two factors function in a tightly coupled manner during the initiation of formation of the open DNA structure. XPC being the most probable primary recognition factor could bring the TFIIH onto the DNA where the other factors would bind and perform opening of the DNA around the lesion. Alternatively, TFIIH could participate in lesion recognition along with XPC-hHR23B complex as observed in bacterial damage recognition with the UvrA$_2$B complex (Orren and Sancar 1990; Friedberg et al. 1995). It is very interesting to note that proteins like RPA, XPA and ERCC1-XPF are more abundant in HeLa cell extracts than XPC or XPG, but TFIIH complex is found interacting with the latter two NER factors in a higher percentage (see Appendix 3).

Additionally one cannot discard the possibility of the existence of fully assembled complexes containing factors involved in the first steps of NER. According to this study these would be less then 10% of the total NER proteins present in the cell (the amount of TFIIH complexed with XPC is only 8%). However they could be able to work as a "repaiosome", performing recognition, open complex formation and dual incision as a fully functional complex upon recruitment of RPA complex.

It is also possible that many different combinations of interactions may exist in the nucleus of mammalian cells. The encounters between these complexes and DNA damage would encourage the formation of new interactions (for instance with heterotrimeric RPA) and strengthen the previously weaker interactions. It is important to note that during the present studies, all the interactions were studied in the absence of DNA or damage induction of the cells.
In this chapter, the strongest and weakest functional interactions between TFIIH and other NER factors have been shown. I detected fully functional complexes \textit{in vitro}, at mild salt concentrations that only need the addition of recombinant RPA in order to be able to perform the dual incisions characteristic of NER. Mechanistically this has some implications, particularly regarding the strong interaction between XPC and TFIIH complexes. Most of all, I have demonstrated the existence of functional interactions between many factors involved in dual incision formation during NER. If these interactions exist stably in the nucleus of mammalian cells or they just reveal transient interactions still remains to be studied.
In recent years, much progress has been made in our understanding of eukaryotic nucleotide excision repair. Various *in vitro* systems to study the NER reaction have been established and the main components of eukaryotic nucleotide excision repair have been identified (see Introduction). Here I summarise the results presented in chapters 4 to 6 and discuss them in light of the overall mechanism of nucleotide excision repair.

7.1 The minimal set of factors involved in nucleotide excision repair *in vitro*

During the studies reported in this thesis I have defined the minimal set of factors involved in nucleotide excision repair of naked DNA *in vitro* by using only recombinant factors for the incision stage, and a mixture of recombinant factors and purified human proteins for the repair synthesis stage. A minimum of 26 polypeptides are necessary for the full NER reaction: RPA complex (three subunits), XPA (1), XPC-hHR23B (2), ERCC1-XPF (2), XPG (1), TFIH complex (minimum of six subunits), PCNA (a homotrimer), RFC (five subunits), pol δ/ε (two subunits each) and ligase I. Further proteins may be required to deal with the additional level of complexity conferred by the chromatin structure of genomic DNA relative to the naked DNA substrate. Candidates to deal with this higher level of repair complexity in humans may include UV-DDB, the human homologues of Rad7-Rad16 and human MMS19.

UV-DDB protein is the candidate for the primary defect in XP-E cells (Rapic-Otrin *et al.* 1998). UV-DDB has been detected in cell extracts as an activity that preferentially binds damaged DNA, with a particular affinity for
(6-4) photoproducts in UV-irradiated DNA (Feldberg and Grossman 1976; Reardon et al. 1993). This activity has been purified as a complex with two subunits of 127 kDa (p127) and 48 kDa (p48) (Keeney et al. 1993) that when microinjected into XP-E cells, lacking UV-DDB activity, substantially correct the NER defect (Keeney et al. 1994; Rapic-Otrin et al. 1998). Mutations in p48 subunit are associated with XP-E in patients lacking this damage binding activity (Nichols et al. 1996; Hwang et al. 1998). UV-DDB is not a core component of the mammalian NER machinery (this thesis and (Aboussekhra et al. 1995)), but may have a specific role in repair of chromosomal DNA. Binding studies of this protein to chromatin, by nuclear fractionation, show that immediately after UV-irradiation it significantly varies the degree of its association, from a looser to a tighter interaction with chromatin (Otrin et al. 1997). Additionally, p48 contains a WD motif that is found in proteins involved in the reorganisation of chromatin (Hwang et al. 1998). An example of such a factor is human CAF-1 that is required for reassembly of chromatin coupled to nucleotide excision repair (Gaillard et al. 1996; Gaillard et al. 1997). Recently, it has also been reported that XP-E cells are defective in global genome repair of CPDs and that levels of p48 mRNA in normal primary fibroblasts increase after DNA damage in a p53 dependent manner (Hwang et al. 1999).

In Saccharomyces cerevisiae, Rad7-Rad16 complex is required for nucleotide excision repair of transcriptionally inactive DNA (Waters et al. 1993; Verhage et al. 1994; Wang et al. 1997). When incision intermediates were trapped in vivo in a cdc9ts mutant strain, rad7 and rad16 mutants were observed to be proficient for NER-dependent incision in repressed loci like HMLα (Reed et al. 1998). This phenotype led to the proposal of a model whereby Rad7-Rad16 complex is involved in postincision events during nucleotide excision repair. These events could be related with excision of the damaged fragment. The transcription machinery could have a role on the excision of the damaged fragment or any other postincision event during...
transcription coupled repair and therefore the involvement of Rad7-Rad16 would only be required for transcriptionally inactive genes. However, Rad7-Rad16 has also been reported to have high affinity and specificity for UV-damaged DNA (Guzder et al. 1997). For this reason, another model was proposed where this complex is involved in coupling damage recognition to the assembly of other NER factors at the site of damage (Guzder et al. 1998b). This agrees with the observation that Rad16 might also have a role in the repair of the transcribed strand of active genes (Teng et al. 1997).

*Saccharomyces cerevisiae* Mms19 has been isolated, purified and migrates in SDS-polyacrylamide gels as a 104 kDa peptide (Lauder et al. 1996). Mms19 appears to be involved in transcription and NER as *mms19Δ* cells are deficient in transcription-coupled repair and global genome repair (Lombaerts et al. 1997). Addition of purified Mms19 does not stimulate the incision of UV-damaged DNA, but addition of purified TFIIH corrects the transcriptional defect also found in these cells. However, Mms19 is not a component of TFIIH neither it appears to be associated with this factor, suggesting an involvement of Mms19 in a process upstream of TFIIH (Lauder et al. 1996; Lombaerts et al. 1997).

Any of these factors might have a role both in the steps leading to the excision of damage and in regulation of NER *in vivo*. Further studies are necessary in order to clarify the roles of these proteins in NER. We currently do not know if knowledge of NER *in vitro* correlates exactly with the *in vivo*, cellular situation. It is known that NER is sensitive to chromatin structures because this reaction is inhibited in the presence of DNA containing nucleosomes (Wang et al. 1991; Sugasawa et al. 1993). Accessibility of the repair machinery to damaged DNA in chromatin is still matter of current study and the mechanism by which NER locates DNA lesions in chromatin *in vivo* is not known. Most probably, chromatin remodelling factors like the ones involved in both replication and transcription can also take part in NER. Additionally, not only the access of
NER proteins to chromatin is necessary, but also nucleosome reassembly after repair synthesis. A factor also involved in chromatin remodelling after replication, CAF-1, was shown to be necessary for repair associated chromatin formation (Gaillard et al. 1996; Gaillard et al. 1997). More studies like these are necessary to investigate the intricate relationship between NER and chromatin remodelling. And more \textit{in vivo} studies, as well as the study of all the mouse knock-out models will clarify the subject of \textit{in vivo} NER and its implications.

7.2 The role of CAK in nucleotide excision repair

Nucleotide excision repair can be reconstituted \textit{in vitro} using a core TFIIH complex without the CAK subcomplex (chapter 4). When added back to reactions reconstituted with pure recombinant factors, recombinant CAK inhibits the excision of damaged products, in the presence of an ATP regenerating system. Essentially, CAK can inhibit NER \textit{in vitro} when the ATP levels are maintained at a high level. Since in the absence of an ATP regenerating system CAK has no effect on the dual incision activity of TFIIH, it is probably the activity of CAK which is inhibiting the reaction rather than simply its physical presence. The inhibition of NER is most likely due to the phosphorylation of some factor involved in the reaction, leading to a decrease in its activity. These results are in agreement with previous work from Rick Wood’s laboratory that showed that phosphatase inhibitors could inhibit NER \textit{in vitro} (Ariza et al. 1996). Further studies of the phosphorylation pattern and activity of the various dual incision factors are necessary in order to find out the mechanism of this inhibition.

CAK was first isolated as a Cdk-activating kinase and is involved in cell-cycle regulation by phosphorylation (Fesquet et al. 1993; Poon et al. 1993; Tassan et al. 1994). The presence of CAK in TFIIH led to the suggestion that CAK might also have roles in transcription regulation (Feaver et al. 1994;
It was shown that the levels of all three subunits of CAK and their kinase activity stay constant over the eukaryotic cell cycle and are not affected by UV-induced DNA damage (Tassan et al. 1994; Adamczewski et al. 1996). However, when associated with TFIIH, CAK activity does diminish after UV-damage (Adamczewski et al. 1996). This is in agreement with a regulation of transcription and repair via TFIIH/CAK. A possible mechanism is CAK autophosphorylation negatively regulating the TFIIH activity. Induced phosphorylation of Cdk7 results in inhibition of the TFIIH-associated kinase and transcription activities as shown both by analysing inactive mitotic TFIIH complex and by inhibiting TFIIH by use of phosphatase inhibitors (Akoulitchev and Reinberg 1998). In human cells, the checkpoint response to ionising radiation involves DNA-PK and ATM, but the UV induced response is still uncertain (reviewed in (Wang 1998)). NER is most probably regulated in a cell-cycle dependent manner and highly controlled when cells are subjected to DNA damage. CAK may be involved in cell-cycle signalling and have a role in regulating transcription and the activity of NER proteins. Independently of the direct involvement of CAK in checkpoint signalling, more studies are necessary to study the pattern of phosphorylation of NER factors and its relation to their activity.

7.3 The role of XPD in nucleotide excision repair

The helicase activity of XPD is necessary for NER but dispensable for transcription (chapter 5). I have demonstrated that mutations in XPD can lead either to TFIIH which is completely inactive in NER or to TFIIH that still has residual activity in dual incision formation. Differences in NER activity of these mutant TFIIH complexes can be correlated with the site of the causative mutation and also with the pattern of interactions between mutant XPD and p44 subunit of TFIIH. However, the activities of TFIIH complexes from XP-D mutant cells do not correlate with the UDS levels
observed upon UV-irradiation and reported in the literature. I demonstrated that these high UDS levels cannot be attributed to NER specific incisions of the damaged DNA duplex. Repair synthesis of a cisplatin damaged DNA substrate containing one single defined lesion also failed to show any damage specific DNA synthesis. The high UDS levels detected in XPD cells still remain a mystery. Perhaps this problem should be approached by analysis within cells. Li and Waters (Li and Waters 1996; Teng et al. 1997) developed a method that allows looking at damage dependent incisions and repair at the level of a specific gene. Maybe this method can be utilised to study incisions in the genome of XPD cells.

### 7.4 NER protein complexes

Once the minimal set of proteins involved in NER was demonstrated to be RPA, XPA, XPC-hHR23B, ERCC1-XPF, XPG and TFIIH complex I investigated the main interactions between these factors. Most of the previously accumulated knowledge was related to individual protein-protein interactions. Some studies have reported the existence of a repairosome that contains all factors necessary for NER, but very little information existed on the functionality of these interactions and their relative strengths.

In chapter 6 of this thesis I investigated the strength of interactions between the core NER factors involved in dual incision formation. The strongest interactions found were between TFIIH and XPC complexes. Less than 10% of TFIIH could be found complexed with XPA, XPC-hHR23B, ERCC1-XPF, and XPG. The strongest interaction was between TFIIH complex and XPC-hHR23B. These complexes were not active in dual incision formation, but upon addition of recombinant RPA their dual incision activity was restored.
Do these results represent strong affinities that give rise to continually maintained higher order complexes within the cell? Or do the factors largely exist separately in cellular environment? Methods such as immunoprecipitation or affinity chromatography can reveal the potential for proteins to interact with one another, but contacts between the factors may not occur generally, but only when they are acting together on damaged DNA during repair. I have measured the proportion of total repair proteins present in immunoprecipitated complexes, and the proportion of functional complexes active in NER. These quantitations are not precise, but provide some interesting background for analysis. My results provide little evidence for a large pool of soluble reairosome in vitro. Only less than 10% could be considered to be a preformed reairosome under the conditions used. However, the interactions exist since it was possible to "pull down" active TFIIH, XPA, XPC-hHR23B, XPG and ERCC1-XPF together. The proteins involved in these higher order complexes detected are active in dual incision formation. Proportionally, their in vitro activity upon addition of RPA is comparable with the activity of the recombinant factors added together to the damaged DNA substrate. Most importantly, what we can conclude from this study is that there is a very strong interaction between XPC-hHR23B complex and TFIIH. More than a third of all the cellular XPC is detected in a TFIIH bound fraction. It would be of great interest to know which components of XPC-hHR23B and TFIIH mediate this interaction in human cells. In S. cerevisiae it is known that Rad23 interacts directly with TFB1 and Rad25 subunits of TFIIH.

The implications of this interaction for the damage recognition step are also very important. TFIIH interacts strongly with XPC-hHR23B and the latter complex has been implicated in DNA damage recognition (Sugasawa et al. 1998). TFIIH is involved in open complex formation, but for this to happen the presence of both RPA and XPA are also needed (Evans et al. 1997b; Mu et al. 1997). Additionally, a complex of XPC-hHR23B, XPA, RPA,
TFIIH and XPG has been reported to have much higher damage specificity than either of its components alone. XPC-hHR23B could be an initial damage recognition protein that, via its strong interaction with TFIIH, creates a protein DNA complex comprising all the factors involved in dual incision formation at the damaged site. Alternatively, the cell nucleus may already contain about 10% of preformed complexes containing all incision factors ready to act on DNA upon damage induction.

More quantitative studies of the distribution of NER proteins in the nucleus of cells are needed. It seems that most basic DNA processes, such as replication and transcription are concentrated within the nuclear matrix forming so-called DNA replication/transcription factories (reviewed in (Cook 1999)). These factories provide a good environment for interactions between these processes. Does nucleotide excision repair show the same behaviour? It is yet unclear if this is indeed so. Different approaches could be used in order to systematically address this hypothesis. The investigation of the amounts of NER proteins attached to the nuclear matrix may be the starting point for such analysis. Also, more in vivo studies are required which directly look at proteins and their movements inside the nucleus of cells (such as (Houtsmuller et al. 1999)).

One of the major topics of interest in the near future will probably be the coordination of other cellular processes with nucleotide excision repair. It is of major interest to identify the connections between DNA repair, transcription regulation, cell cycle progression, replication and recombination as well as to elucidate the factors mediating such regulation. The overlap between all repair processes is also starting to be unravelled. For example, XPG is known to interact and stimulate hNTh1, a protein involved in BER (Cooper et al. 1997; Nouspikel et al. 1997; Klungland et al. 1999). In S. cerevisiae components of the NER pathway were found to co-immunoprecipitate with Msh2, suggesting that mismatch repair proteins
may exist in a complex with some NER/recombination proteins (Bertrand et al. 1998). Some of these interactions may be due to the involvement of Rad1, Rad10, Msh1, Msh3 in SSA recombination during gene conversion (Paques and Haber 1997).

7.5 Implications of NER in human disease

There are several rare human inherited disorders associated with nucleotide excision repair and these have been referred to throughout this thesis. Both XP, CS and TTD can arise by mutation of the genes encoding NER proteins. Most importantly, nucleotide excision repair is related to human cancer. Not only defects in NER proteins can give rise to cancer prone repair syndromes, but selective inhibition of NER could prove life saving during cancer treatment.

Cisplatin is a chemotherapeutic drug used with varying success for the treatment of human cancers. During cancer treatment a significant problem arises when tumours become resistant to the drug. The basis for the cytotoxic effects of cisplatin is thought to be mediated through formation of cisplatin-DNA adducts which may inhibit DNA replication and/or transcription (reviewed in (Zamble and Lippard 1995)). More recently, the cisplatin-sensitivity of testicular germ cell tumours has been attributed to a reduced level of XPA protein (Köberle et al. 1999). These observations have very important implications for cancer treatment. They suggest that specific inhibition of a repair protein could sensitise tumours to cisplatin, enabling a more effective treatment of these tumours. Additionally, in vitro analysis of tumour cells might prove to be a tool for a better choice of chemotherapy. In summary, the more we know about NER and the way it works in vivo, the closer we will be to better cancer treatments.
REFERENCES


Lehmann, A., S. Kirk-Bell, C. Arlett, M.C. Paterson, P.H.M. Lohman, E.A. de Weerd-Kastelein, and D. Bootsma. 1975. Xeroderma pigmentosum cells with normal levels of


References


Reardon, J.T., A.F. Nichols, S. Keeney, C.A. Smith, J.S. Taylor, S. Linn, and A. Sancar. 1993. Comparative analysis of binding of human damaged DNA-binding protein (XPE) and *Escherichia coli* damage recognition protein (UvrA) to the major ultraviolet photoproducts - t[c,s]t, t[t,s]t, t[6-4]t, and t[dewar]t. *J. Biol. Chem.* **268**: 21301-21308.

Reed, S.H., S. Boiteux, and R. Waters. 1996. UV-induced endonuclease III-sensitive sites at the mating-type loci in *Saccharomyces cerevisiae* are repaired by nucleotide excision-repair - Rad7 and Rad16 are not required for their removal from HMLα. *Mol. Gen. Genet.* **250**: 505-514.


References


References


References


References


APPENDIX 1
Recombinant ERCC1-XPF purification

**Construct:** pET30bERCC41 (transformed into strain FB810) recA derivative of BL21 (DE3) (Benson *et al.* 1994)).

Twelve litres of *E. coli* containing this construct were grown in Luria-Broth media+Kanamycin and lysates were prepared by sonication.

ERCC1-XPF complex was purified according to the following scheme.

```plaintext
12 l E. coli  
sonication  
Phosphocellulose  
1 M KCl  
Ni-NTA  
1 M KCl  
+  
5mM imidazole  
MonoQ  
400mM  
150mM  
250mM  
ERCC1-XPF
```
## APPENDIX 2

### Buffer compositions of pure proteins

#### RPA
- 30 mM Hepes pH 7.8
- 250 mM NaCl
- 0.25 mM EDTA
- 0.25% Inositol
- 0.01% NP40
- 1 mM DTT

#### XPA (CJ prep 450ng/ml)
- 25 mM Hepes pH 7.8
- 500 mM KCl
- 0.4 mM EDTA
- 10% glycerol
- 125 mM PO4^2-
- 1 mM DTT

#### XPChHR23B (bac recomb)
- 25 mM Tris
- 300 mM NaCl
- 0.5 mM EDTA
- 12.5% glycerol
- 0.01% TritonX
- 2 mM DTT

#### XPChHR23B (HeLa prep)
- 25 mM Hepes pH 7.8
- 100 mM KCl
- 1 mM EDTA
- 10% glycerol
- 0.01% NP40
- 0.1 mM PMSF
- 1 mM DTT

#### XPG (bac recomb prep III)
- 25 mM Hepes pH 7.5
- 80 mM NaCl
- 1 mM EDTA
- 50% glycerol
- 0.04% NP40
- 1 mM DTT

#### XPG (bac recomb prep IV)
- 25 mM Hepes pH 7.5
- 50 mM KCl
- 0.1 mM EDTA
- 50% glycerol
- 0.02% NP40
- 1 mM DTT

#### ERCC1-XPF (EF2)
- 25 mM Hepes pH 7.8
- 300 mM NaCl
- 1 mM EDTA
- 10% glycerol
- 1 mM DTT
**TFIIH (Hep, Hap and recombinant)**
- 50 mM Tris pH 7.6
- 50 mM KCl
- 0.1 mM EDTA
- 20% glycerol
- 0.5 mM DTT

**Dialysis Buffer**
- 25 mM Hapes pH 7.8
- 100 mM KCl
- 1 mM EDTA
- 12 mM MgCl₂
- 17% glycerol
- 2 mM DTT
Appendix 3
Immunoblot quantifications

Quantifications of the amounts of each NER factor present in the immunoprecipitate were performed by immunoblot. Protein bands from immunoblots were quantified using NIH image. Titrations were done using HeLa total cell extracts and standard amounts of recombinant RPA, XPA, XPC-hHR23B, ERCC1-XPF and XPG. TFIIH quantifications were done in a slightly different way, since no standard concentration exists for the various subunits of this complex. Therefore, the values calculated by Kimura et al. for the total number of TFIIH molecules per cell were used as standard (Kimura et al. 1999).

- TFIIH complex quantification (anti-p62 immunoblot):

![Graph](image)

According to (Kimura et al. 1999), there are $1.1 \times 10^5$ TFIIH molecules per HeLa cell. 1 μl of beads corresponds to protein immunoprecipitated from $1.5 \times 10^6$ cells, therefore assuming that 100% of TFIIH is being "pulled-down", there are $1.7 \times 10^{11}$ TFIIH molecules per μl of beads and that corresponds to 270 fmol. Using the graph, it is estimated that 1.5 μl of TFIIH
Hep contain $3 \times 10^{11}$ molecules or 500 fmol of TFIIH complex.

- **XPA quantification:**

  ![Graph](image)

  - Density of XPA band corresponding to 20 µl of beads = 931
  - fmol XPA per µl of beads = 15

- **XPG quantification:**

  ![Graph](image)

  - Density of XPG band corresponding to 20 µl of beads = 754
  - fmol XPG per µl of beads = 35
- ERCC1 quantification:

![Graph showing ERCC1 quantification](image)

\[ y = -255.92 + 4724.5x \quad R^2 = 0.940 \]

- Density of ERCC1 band corresponding to 20 μl of beads = 469
- fmol ERCC1 per μl of beads = 5

- XPF quantification:

![Graph showing XPF quantification](image)

\[ y = -66.775 + 1576.4x \quad R^2 = 0.962 \]

- Density of XPF band corresponding to 20 μl of beads = 591
- fmol XPF per μl of beads = 15

The ratio of ERCC1 and XPF in ERCC1-XPF is 1:1. From these calculations the values being immunoprecipitated with TFIIh are different. Since the density value for the XPF band is more within the range of the regression, it
was assumed that the amounts of ERCC1-XPF were 15 fmol.

- **XPC quantification:**

\[ y = 50.000 + 2759.7x \quad R^2 = 0.998 \]

- Density of XPC band corresponding to 20 μl of beads = 1187
- fmol XPC per μl of beads = 22

- **hHR23B quantification:**

\[ y = 144.50 + 1712.7x \quad R^2 = 0.955 \]

- Density of hHR23B band corresponding to 20 μl of beads = 334
- fmol hHR23B per μl of beads = 10

XPC-hHR23B is a 1:1 complex. Again, the value for XPC titration is more
within the linear region of the graph and for this extrapolation the value of
fmol for the XPC-hHR23B complex was assumed to be 20 fmol.

-Molecules per cell

Using the previous graphs and immunoblotting titrations presented in
chapter 6 an attempt could also be made to calculate the different amounts
of NER factors per cell.

<table>
<thead>
<tr>
<th>NER factor</th>
<th>Number of molecules per single HeLa cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPA</td>
<td>200000</td>
</tr>
<tr>
<td>XPA</td>
<td>200000</td>
</tr>
<tr>
<td>XPC</td>
<td>25000</td>
</tr>
<tr>
<td>hHR23B</td>
<td>250000</td>
</tr>
<tr>
<td>ERCC1</td>
<td>100000</td>
</tr>
<tr>
<td>XPF</td>
<td>100000</td>
</tr>
<tr>
<td>XPG</td>
<td>80000</td>
</tr>
<tr>
<td>TFIIH*</td>
<td>110000*</td>
</tr>
</tbody>
</table>

* - number of TFIIH molecules per cell was calculated in (Kimura et al. 1999)

- Comparison of XPC-hHR23B molecule totals

According to (van der Spek et al. 1996) there are 4-8 x 10^4 molecules of
XPC and 2-4 x 10^5 of hHR23B per HeLa cell.

36% of XPC corresponds to 7000-14000 molecules of XPC being "pulled
down" with TFIIH per cell (110000) and that corresponds to a total of 17.5-35
fmol of XPC complexed with 270 fmol of TFIIH per 1.5 x 10^6 HeLa cells or 1
µl of beads. About 10% of the total estimated TFIIH is complexed with XPC.
1% of total hHR23B corresponds to 2000-4000 molecules. This corresponds to a total of 5-10 fmol being immunoprecipitated with 270 fmol of TFIIH.

- Comparison of RPA molecule totals

According to (Wold 1997) the number of RPA complexes per cell is $3 \times 10^4 - 2 \times 10^5$. The value calculated in this study was $2 \times 10^5$ which is within the same range.

The quantifications presented in this Appendix are only rough estimates and not absolute values. Their only purpose is to compare the amounts of various proteins present in the same immunoprecipitate.
APPENDIX 4

Curriculum vitae

Education

1989-1994 undergraduate studies

- **July 1994** - Degree in Applied Chemistry - Biotechnology, by Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal, with the final classification of 16/20.
- **1993/1994** - Graduation degree project on *Purification of VP73 protein of African Swine Fever Virus (ASFV) and production of monoclonal antibodies* under the supervision of Doutor Carlos Sinogas and Doutor João Vasconcelos Costa, Instituto Gulbenkian de Ciência, Oeiras, Portugal.

1994-1999 graduate studies

- **1994/1995** - First year of Ph.D. studies at Instituto Gulbenkian de Ciência (Programa Gulbenkian de Doutoramento em Biologia e Medicina), under the coordination of Prof. António Coutinho and Prof. Alexandre Quintanilha. Courses covering Molecular, Cellular and Developmental Biology, Genetics and Biochemistry.
- **1995-1999** - Laboratory studies for Ph.D., Imperial Cancer Research Fund, Clare Hall Laboratories, UK, under the supervision of Dr. Richard D. Wood, funded by the Portuguese Programa Gulbenkian de Doutoramento em Biologia e Medicina, Fundaçao Ciência e Tecnologia and the Imperial Cancer Research Fund.

Publications

Araújo, S. J. and Wood R. D., Nucleotide excision repairasomes and protein complexes, Mutation Research (in press, 1999)


**Oral presentations**

September, 1996 - Pre-Doctoral Meeting of the Biochemical Society, Queen Mary and Westfield College, London, UK.
April, 1997 - ICRF Clare Hall Internal Seminar, London, UK.
January 1999 - Invited lecture on DNA repair mechanisms at Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal.
February 1999 - Keystone Sympsoias on Molecular Mechanisms in DNA Replication and Recombination, Taos, New Mexico, USA.

**Poster presentations**

