DUAL INVOLVEMENT OF TFIIH IN DNA REPAIR AND TRANSCRIPTION

DUBBELE BETROKKENHEID VAN TFIIH IN DNA HERSTEL EN TRANSCRIPTIE

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Voor Joyce en Dennis

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LIST OF ABBREVIATIONS

(6-4)PP	pyrimidyne(6-4)pyrymidone
(A)AF	(acetyl)aminofluorene
CHO	Chinese hamster ovary
CS	Cockayne's syndrome
CPD	Cyclobutane pyrimidinedimer
CTD	Carboxy-terminal domain
DNA	Deoxyribonucleic acid
HA	Hemagglutinin
NER	Nucleotide excision repair
(d)NTP	(deoxy)Nucleotide triphosphate
RNA	Ribonucleic acid
TFIIH	Transcription factor IIH
TTD	Trichothiodystrophy
UDS	Unscheduled DNA synthesis
UV	Ultraviolet
WCE	Whole-cell extract
XP	Xeroderma pigmentosum

GENERAL INTRODUCTION

DNA REPAIR MECHANISMS

The carrier of genetic information, deoxyribonucleic acid (DNA), appears at the macromolecular level as an extremely stable molecule as it is faithfully duplicated and transmitted from mother to daughter cells. At the molecular level, however, DNA is subject to continuous attack, even under physiological conditions and temperature (1). Such endogenous damage is mainly due to reactivity of the base moiety and not of the sugar-phosphate backbone. The main lesions introduced are due to hydrolysis, e.g. of the *N*-glycosidic bond resulting in abasic sites, oxidation reactions, and non-enzymatic alkylation. In contrast to these modifications, DNA damage can also be induced by environmental factors, such as chemicals that are reactive with base groups, or physical agents, such as UV or ionizing radiation. Both spontaneous and induced DNA damage can interfere directly with DNA metabolism and result in abnormal cell behavior, cell death, or induction of permanent alterations of the genetic material.

Several distinct DNA repair mechanisms have been described that prevent the effects of DNA damage in a variety of organisms including *E. coli*, the unicellular lower eukaryote *S. cerevisiae*, and higher eukaryotes. These pathways utilize different strategies for recognition and removal of lesions from DNA, each with its own damage specificity (2). Together, these DNA repair mechanisms can eliminate virtually any type of DNA injury. For instance, spontaneous damage is mainly removed by the base excision repair machinery. This repair pathway employs a set of distinct DNA glycosylases, each recognizing specific, or a small class of structurally related, base lesions. After recognition, the damaged base is removed by enzymatic hydrolysis of the *N*-glycosidic bond. The resulting apurinic/apyrimidinic site is subsequently processed by additional enzymes, including a DNA polymerase and a DNA ligase (3,4). A different strategy is adopted by nucleotide excision repair (NER). Numerous helix-distorting lesions are eliminated from DNA by this multi-enzyme pathway, including UV-induced photoproducts. Rather than recognizing specific lesions, the NER machinery somehow senses alterations in the DNA structure and removes a damage as part of an oligonucleotide (5,6). Other repair systems include direct reversal of a methylated base by a single protein, and the repair pathways that deal with double-stranded DNA breaks induced by ionizing radiation or chemicals: homology-dependent recombination and end-joining (7,8).

The importance of DNA repair mechanisms in humans and their role in the prevention of cancer is illustrated by the occurrence of inherited diseases associated with defective repair. Xeroderma pigmentosum, a rare autosomal recessive disease, is the prototype DNA repair disorder caused by malfunctioning of NER (9). The

clinical symptoms include extreme sun-sensitivity and >2000-fold elevated incidence of skin cancer. Related syndromes are Cockayne's syndrome (CS) and the photosensitive form of trichothiodystrophy (TTD), which are also associated with defective NER (9). Besides a marked UV-sensitivity, the main characteristics of CS comprise severe neurodevelopmental abnormalities and symptoms of premature aging, while TTD displays in addition brittle hair and nails due to impaired expression of sulphur-rich matrix proteins. These three syndromes are genetically heterogeneous encompassing at least ten complementation groups. Considerable overlap and clinical heterogeneity has been identified in a subset of these complementation groups, exemplified in case of XP-D, which harbors patients suffering from XP, combined XP and CS, or TTD.

NER is functionally linked with several different cellular processes, for instance, cell cycle control and RNA polymerase II transcription. First clues for the connection with transcription came from the observations that certain lesions in actively transcribed genes are repaired faster than lesions present in inactive loci. Furthermore, it became apparent that some NER factors are essential for viability due to a requirement in RNA polymerase II transcription.

TRANSCRIPTION BY RNA POLYMERASE II

Three distinct nuclear DNA-dependent RNA polymerases are present in eukaryotic cells each devoted to a specific class of genes. RNA polymerase I is dedicated to transcription of the large ribosomal RNA precursor (class I). Class III genes encoding many structural RNA molecules including transfer-RNAs are transcribed by RNA polymerase III. Messenger RNA from protein-coding genes and several small-nuclear RNAs (class II) are synthesized by RNA polymerase II. Classical class II promoters contain a conserved TATA consensus sequence at 25-30 base pairs upstream of the transcription start site, downstream elements, including a poorly conserved initiator sequence, and upstream promoter elements. The ability to recognize a class II promoter sequence is not intrinsic to the RNA polymerase II molecule itself, but requires a set of additional proteins, which are referred to as basal transcription factors of RNA polymerase II (TFII). This set of protein factors was identified by fractionation of cell extracts capable of promoter-specific transcription and named according to the chromatographic fraction and order of identification: TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (10,11).

TFIIH: A BASAL TRANSCRIPTION FACTOR INVOLVED IN NUCLEOTIDE EXCISION REPAIR

Human TFIIH is a protein complex consisting of nine subunits. Protein sequencing of the subunits identified the largest two subunits of TFIIH as the products of the previously cloned genes defective in XP-B and XP-D cells, revealing a dual

function for TFIIH in NER and RNA polymerase II transcription (12,13). The protein complex possesses several enzymatic activities. The largest subunits, XPB and XPD, have DNA-dependent adenosine triphosphatase (ATPase) and DNA helicase activities, implicating TFIIH in an ATP-dependent helix unwinding step that occurs both in NER and transcription initiation by RNA polymerase II (14,15). CDK7 is a kinase catalytic subunit that is able to phosphorylate the C-terminal domain of the largest subunit of RNA polymerase II (16).

SCOPE OF THE THESIS

The aim of the work described in this thesis is to further characterize the precise role of TFIIH in NER and RNA polymerase II transcription. Attention was largely focussed on two questions: (i) what is the composition of TFIIH in DNA repair and transcription, and (ii) what is the role of the helicase subunits of TFIIH, XPB and XPD? In **chapter 1**, a review of current literature on the function of TFIIH in DNA repair and transcription is presented. The main findings outlined in the experimental part of the thesis are also integrated and discussed in this chapter, which will focus on the mechanisms in mammalian cells, with a strong emphasis on the biochemical aspects involved. **Chapter 2** describes a relatively simple, two-step procedure that allows the purification of TFIIH without the need for extensive chromatography, facilitating a detailed analysis of the composition and biochemical activity of TFIIH. **Chapter 3** describes a modification of this procedure, which allowed the isolation and characterization of mammalian TFIIH with inactive XPD helicase subunit. **Chapter 4** focusses on the role of TFIIH in NER, describing how TFIIH functionally interacts with several NER factors. This analysis reveals a possible novel function for TFIIH and ATP in the NER reaction, which may involve the enzymatic activity of the XPB and XPD subunits. Finally, **chapter 5** reports on the cDNA cloning, and partial characterization of the human counterpart of yeast MMS19, a factor that is able to regulate TFIIH function (17,18).

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CHAPTER 1

The Multifunctional TFIIH Complex: Dual Requirement in DNA Repair and Transcription

To be submitted for publication

THE MULTIFUNCTIONAL TFIIH COMPLEX: DUAL REQUIREMENT IN DNA REPAIR AND TRANSCRIPTION

Transcription factor IIH (TFIIH) is a high molecular weight multisubunit complex that contains associated DNA-dependent ATPase, DNA helicase, and protein kinase activities. Functionally, the complex has been implicated in both DNA repair and RNA polymerase II transcription. In these processes, TFIIH and its enzymatic activities are utilized during different stages in the reactions. This review will discuss the biochemistry of NER and its relation with RNA polymerase II transcription with emphasis on the pivotal role of mammalian TFIIH.

THE POLYPEPTIDE COMPOSITION OF TFIIH

TFIIH is one of five basal transcription factors required by RNA polymerase II for promoter-specific transcription of class II genes *in vitro*, which include all protein-coding genes. The multifunctional complex is composed of nine subunits (1-3) (see Table 1), and has a dual role in both RNA polymerase II transcription and nucleotide excision repair (NER) (4-8). The two largest subunits, XPB and XPD, were originally identified as gene products defective in mammalian NER mutants (9,10) and provide TFIIH with two DNA-dependent ATPase and DNA helicase activities with opposite polarity when studied as isolated recombinant proteins (11,12). Furthermore, the complex comprises a kinase activity, which resides in the CDK7 subunit and is able to phosphorylate the tandem heptapeptide repeat of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (13-16). CDK7, Cyclin H and MAT-1 are integral subunits of TFIIH, but also form a separate trimeric CDK-activating kinase (CAK) complex. CAK is implicated in the activation by phosphorylation of cyclin-dependent kinases *in vitro* (15-18), as well as *in vivo* in *S. pombe* and *Drosophila* (19-21). In contrast, no physiological function in cell cycle progression has been observed for the homologs in *S. cerevisiae* (22), in which organism physiological CAK activity resides with a single protein that does not show homology with human CDK7 (23,24). No enzymatic function has been associated with the other TFIIH subunits p62, p52, p44, and p34 subunits (1,25,26), but p44 is able to stimulate the DNA helicase activity of recombinant XPD (11).

Yeast genetics demonstrated that all subunits are essential for viability (27). Furthermore, temperature-sensitive alleles encoding the Ssl2/Rad25 subunit (the homolog of human XPB), Rad3 (XPD), Tfb1 (p62), and Ssl1 (p44) cause conditional lethality, due to a transcription defect, and UV-sensitivity at the permissive temperature (28-30). A C-terminal deletion of Tfb2 (p52) renders yeast cells UV-sensitive (31). These observations underscore the dual involvement of TFIIH in DNA repair and transcription.

THE MECHANISM OF NUCLEOTIDE EXCISION REPAIR

Nucleotide excision repair (NER) is an important DNA repair mechanism that is able to remove many different DNA lesions, including cyclobutane pyrimidine dimers (CPD) and pyrimidine(6-4)pyrimidone lesions ([6-4]PP), the two main UV-induced photoproducts (32-34). Thus, it prevents the persistance of lesions in the genome, which can interfere with cell metabolism and induce mutations leading to permanent alterations of the genetic material. In humans, defects in NER are associated with the inherited syndromes xeroderma pigmentosum (XP), Cockayne's syndrome (CS), and a photosensitive form of trichothiodystrophy (TTD). Cell fusion experiments have identified ten complementation groups in these partially overlapping syndromes: six in XP (XP-A, and XP-C through XP-G), three in combined XP/CS (XP-B, XP-D, and XP-G), two in CS (CS-A and CS-B), and three in TTD (XP-B, XP-D, and TTD-A). All the corresponding genes have been identified, except in the case of TTD-A (35). Two sub-pathways constitute NER: global genome NER and transcription-coupled NER (36,37). The NER defect in XP-C is restricted to the global genome NER pathway (38), while CS-A and CS-B cells are specifically deficient in the transcription-coupled pathway (39).

Global genome NER pathway can be divided into four principal steps: (i) damage-recognition, (ii) formation of an open complex which involves melting of the DNA helix, (iii) dual incision of the damaged strand on both sides of the lesion resulting in removal of the lesion as part of a 24-32-mer oligonucleotide, and (iv) gap-filling repair DNA synthesis (Figure 1). While the complete repair reaction can be carried out *in vitro* and requires about 30 polypeptides (40), six protein factors (summarized in Table 1) are required for damage-recognition, open complex formation and dual incision: the XPC-HR23B complex, XPA, RPA, a human single-stranded DNA binding heterotrimer additionally involved in recombination and replication, TFIIH, and the structure-specific endonucleases XPG and ERCC1-XPF (41,42). In the transcription-coupled NER pathway, a DNA damage is detected by the elongating RNA polymerase II complex when it encounters a lesion and does not depend on the XPC-HR23B complex. This repair pathway will be discussed later in this chapter.

Substrate-recognition: initial binding by the XPC-HR23B complex

The extreme versatility of the mammalian NER machinery is illustrated by the wide variety of lesions that are recognized and removed by this repair pathway (Table 2). The initial recognition of lesions in global genome NER is achieved by the XPC-HR23B heterodimer (43). The complex binds directly to a site of DNA damage as evidenced by a distinct DNase-I footprint. Furthermore, the XPC-complex binds with higher affinity to damaged DNA as compared to XPA or a combination of XPA and RPA as assessed in competition assays (43). The

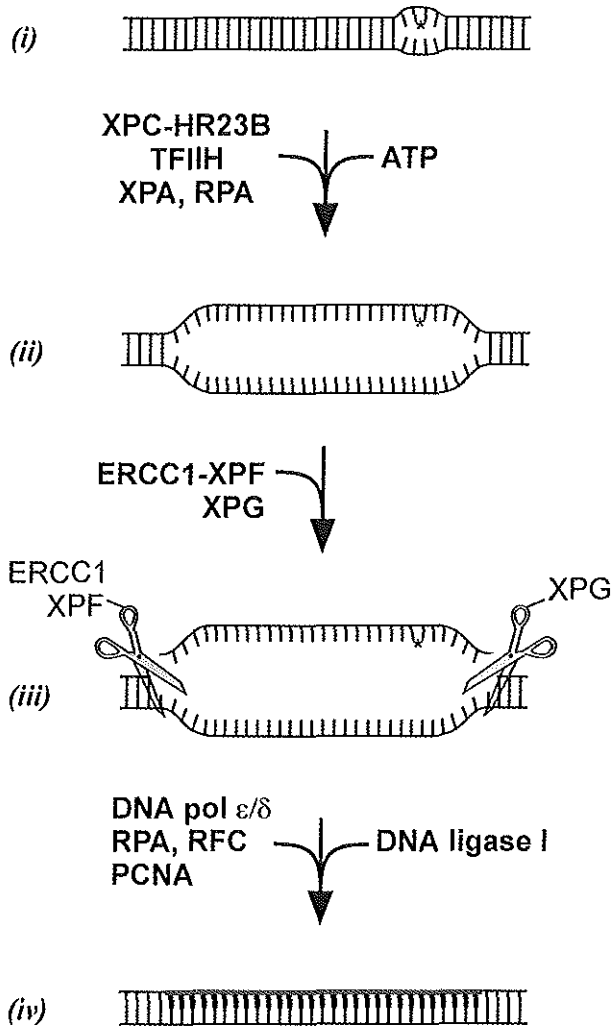

Figure 1. Model for the molecular mechanism of nucleotide excision repair. (i) Lesions recognized by the NER machinery usually cause helical alterations including local base pair disruptions. (ii) After recognition by the XPC-HR23B complex, the DNA around the lesion is converted into an open structure, which requires the concerted action of at least four protein factors, including TFIIH, and ATP. (iii) Dual incision of the damaged strand and removal of the lesion as part of a ~30-mer oligonucleotide requires the action of two structure-specific endonucleases, XPG and ERCC1-XPF, which cut the damaged strand at single strand/double strand junctions at the 3' and 5' side, respectively. (iv) The resulting gap is filled in by the DNA replication machinery. Strand continuity is achieved by a DNA ligase, which restores the original DNA sequence and completes the NER event.

Table 1. Eukaryotic nucleotide excision repair factors required for dual incision.

Human factor	Subunit [a,b]	yeast homolog [c]	Properties of human factor	References
XPA	43	Rad14	Zn-finger; binds preferentially to damaged and single-stranded DNA	57
XPC-HR23B	125 (XPC) 58 (HR23B)	Rad4 Rad23	Complex binds with high affinity and specificity to DNA-damage; binds to double and single-stranded DNA	43,144
RPA	70 32 14		Human single-stranded DNA binding protein; additionally involved in DNA (repair) replication, and homologous recombination	
TFIIH	89 (XPB/ERCC3)	Rad25/Ssl2	DNA-dependent ATPase, 3'-5' helicase	11,12
	80 (XPD/ERCC2)	Rad3	DNA-dependent ATPase, 5'-3' helicase	11,12,145
	62	Tfb1		
	52	Tfb2		
	44	Ssl1	Zn-finger protein; putative DNA-binding	26
	34	Tfb3	Zn-finger protein	
	40 (CDK7)	Kin28	Kinase catalytic subunit; subunit of CAK	14
	36 (Cyclin H)	Ccl1	subunit of CAK	
	32 (MAT1)	Tfb4	subunit of CAK	
ERCC1-XPF	103 (XPF)	Rad1	Structure-specific endonuclease complex	146
	33 (ERCC1)	Rad10		
XPG	133	Rad2	Structure-specific endonuclease	72,74

[a] The numbers refer to the calculated molecular weight of the subunits (kD).
[b] References for identification of these factors in NER are extensively reviewed (35).
[c] References for *S. cerevisiae* genes and proteins are reviewed elsewhere (27,32).

subsequent recruitment of three additional repair factors (XPA, RPA, and TFIIH) results in a complex with higher binding specificity as compared to either factor alone as assayed in electrophoretic mobility shift gel-assays (44). Also, it was demonstrated that the presence of five factors (XPC-HR23B, XPA, RPA, TFIIH, and XPG) in the reaction mixture results in the formation of a complex that displays even higher specificity for damaged DNA (44). Apparently, the latter complex does not contain XPC-HR23B, although the heterodimer is required for its formation (44). Together, these data suggest a model in which XPC-HR23B nucleates the assembly of a pre-incision complex. Additional factors (XPA, RPA, and TFIIH) further increase binding specificity for a site of DNA damage, which maximizes upon association of XPG presumably resulting in a conformation that allows release of XPC-HR23B from the damaged site.

Not all lesions are recognized and removed with equal efficiency. In human cells, for instance, the predominant CPD photoproducts are repaired approximately five times slower as compared to the less frequently formed but more distorting (6-4)PPs (45). This is also reflected in cell extracts where single (6-4)PPs are much more efficiently removed from plasmid DNA as compared to single CPD lesions (46). In general, efficient recognition correlates with increasing local distortion of

the double-helix caused by a lesion (47-51). Interestingly, some DNA lesions are removed in the absence of XPC-HR23B *in vitro*. Both a certain type of cholesterol moiety as well as a CPD located in a 10-20 nucleotide bubble were recognized independently of XPC-HR23B and removed with correctly placed incisions with respect to the lesion (52-54). Thus, it may be speculated that the XPC-complex recruits additional repair proteins, such as XPA, by increasing the sugar-phosphate backbone distortion and/or helix-unwinding in addition to protein interactions. At least one line of evidence is supporting a role for XPC-HR23B in altering the DNA conformation around a lesion. In the absence of hydrolyzable ATP, a $KMnO_4$ hypersensitive site in the vicinity of a DNA damage is observed in incision-defective cell extracts except in those derived from XP-C cells (55).

Table 2. Some substrates for human NER *in vitro* [a].

Adduct/compound	Abbreviation	References
UV irradiation:		147
pyrimidine(6-4)pyrimidone photoproduct	(6-4)PP	46
cyclopyrimidine dimer photoproduct	CPD	46,148
N-acetylaminofluorene	AAF	84
4' hydroxymethyl-4,5',8-trimethylpsoralen	HMT	84,148,149
cis-diammino, dichloroplatinum(II):	cis-platin	148,150
{cis-diamminoplatinum(II)} 1,2- (GpG) intrastrand crosslink		151
{cis-diamminoplatinum(II)} 1,3-(GpTpG) intrastrand crosslink		42,151
2-aminobutyl-1,3-propanediol ("synthetic abasic site")	ABPD	149
O^6-methylguanine	O^6-MeG	149
N^6-methyladenine	N^6-MeA	149
cholesterol linked to 2-aminobutyl-1,3-propanediol		76
G-A/G-G mismatched bases		149
benzo[*a*]pyrene diol-epoxide	BPDE	47,152
8-methoxypsoralen	8-MOP	47
CC-1065		47
anthramycin		47
oxygen free radical-induced lesions		153
Mispaired base(s) containing:		
{cis-diamminoplatinum(II)} 1,2- (GpG) intrastrand crosslink		51
{cis-diamminoplatinum(II)} 1,3-(GpTpG) intrastrand crosslink		51
pyrimidine(6-4)pyrimidone photoproduct		50
C4'-modified backbone modifications:		48
C4'-selenophenyl		48
C4'-pivaloyl		48
C4'-inverted		49
L-deoxyribose substitution		

[a] Note that significant quantitative differences are present in the recognition and removal of the various lesions.

XPA with or without the help of RPA may recognize lesions that are eliminated in the absence of XPC-HR23B. XPA binds preferentially to UV-irradiated or cis-platin treated double-stranded DNA or single-stranded DNA as compared to non-damaged DNA (56,57). However, when short damage-containing oligonucleotides were used, no damage-specificity was observed (58), likely due to XPA affinity for

DNA ends (our unpublished observations). A specific interaction between the 70 and 32 kD subunits of RPA with XPA is well documented (59-61). In the presence of RPA, XPA binds with significantly increased specificity to DNA treated with damaging agents (60,61). Two different deletions of four aminoacids in XPA that result in severely reduced interactions with the 70 kD subunit of RPA exhibit no activity in the NER reaction, suggesting that the interaction between RPA and XPA is essential for NER (61). In addition to an auxialiary role in damage-recognition by increasing the specificity of XPA for DNA damage, RPA alone also displays an increased affinity for UV-irradiated DNA, possibly due to the increased single-stranded character induced by (6-4)PPs (62).

Open complex formation: ATP-dependent DNA unwinding mediated by TFIIH

After lesion recognition by XPC-HR23B, the first protein complex identified consists of XPA, RPA, XPC-HR23B, and TFIIH. The C-terminus of XPA specifically binds to TFIIH (63). In the absence of additional factors, XPA can recruit TFIIH to DNA lesions (64), while in XPA-deficient extracts, stable binding of TFIIH to damaged DNA is not observed (65). These data suggest that XPA is important for recruitment of TFIIH to the damaged site. The complex of four protein factors is minimally required for ATP-dependent DNA unwinding around the lesion (54,55,66). In one report, the local unwinding extends over less than 20 nucleotides with a (6-4)PP lesion located close to the center of the open region (54). In another study, the open region extends over ~25 nucleotides with a 1,3-d(GpTpG) cisplatin crosslink located closer toward the 3' border of the open region (66). In the latter study, the ends of the melted region colocalize with the determined incision sites for this lesion (42,66), which would suggest that the extent of the melted region determines the positions of the incisions.

The conformational changes in the DNA substrate are linked to hydrolysis of the β-γ bond of ATP as the non-hydrolyzable ATPγS and AMP-PNP analogs cannot substitute (54,55). Because TFIIH is the only incision factor that possesses ATP-dependent enzymatic activities and contains an associated DNA unwinding activity (4,67-69), this component is likely to facilitate the formation of the open complex. The ATPase activity of TFIIH may be subject to regulation by other NER proteins. The activity is specifically stimulated in the presence of XPC-HR23B or XPA, the latter of which enhances the rate of ATP-hydrolysis specifically in the presence of damaged DNA (70). Further indications for the involvement of the helicase subunits in the formation of an open region were obtained using cell extracts derived from XP-B or XP-D cells. In several XP-D extracts, full opening is not observed (55). Furthermore, TFIIH with enzymatically inactive XPD does not allow the incision on neither side of a DNA lesion, in agreement with the idea that DNA opening is required for the formation of both incisions (71). In addition, two out of three extracts derived from different XP-B cell lines are defective in the DNA opening

step of the NER reaction (55). An XP-B extract derived from XP11BE cells, however, still displays normal opening, but is defective in the formation of the 5' incision (55). As these results suggest that the primary role of TFIIH is to create an open complex, pre-melted substrates have been tested to bypass the requirement for ATP and/or TFIIH (54). Although some of these heteroduplex substrates do not demand the XPC-complex, the requirement for either ATP or TFIIH cannot be bypassed, suggesting additional functions for TFIIH and ATP hydrolysis distinct from DNA unwinding (54).

Dual incision and release of the damage-containing oligonucleotide

Dual incision requires the presence of the structure-specific endonucleases XPG and ERCC1-XPF (72,73), which make the 3' and 5' incisions, respectively (73,74). XPG enters a nucleoprotein complex formed by XPA, RPA, XPC-HR23B, and TFIIH, which may result in release of the XPC-HR23B heterodimer (44). ERCC1-XPF can only be detected in complexes containing XPG (44). Both XPG and ERCC1-XPF are not essential for open complex formation (54), nor do mutant proteins interfere at this stage (55,66). However, XPG can facilitate the formation of an open region by binding and stabilizing the complex. This auxiliary function of XPG is distinct from its enzymatic activity as mutations in the active-site do not affect this characteristic (54). The incisions are placed asymmetrically around a lesion (42,75) as exemplified for a CPD photoproduct, which is removed by incisions mainly of the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' of the dimer (75). A similar, but not identical, pattern has been observed for other DNA modifications (42,76). The formation of the 5' and 3' incisions are closely coupled in time, and kinetic experiments indicate that the 3' incision is made before the 5' incision (52,55). Moreover, uncoupled 3' incisions are observed in the absence of ERCC1-XPF, but no uncoupled 5' incisions are detected to significant extent in the absence of XPG (52). However, 5' incisions are seen in the presence of active-site XPG mutants, which indicates that the physical presence of XPG and not the 3' nick determines the formation of the 5' incision (77).

At least two factors have been identified that may be involved in regulation of the endonucleolytic activities. RPA appears to play an important role in the coordination of both incisions. It stimulates the formation of nicks by ERCC1-XPF, possibly by increasing the affinity of ERCC1-XPF for its DNA substrate, which depends on the binding polarity of RPA, and/or by stimulating its endonucleolytic activity (78,79). RPA also influences binding of XPG to DNA, which is dependent on the direction of RPA binding (78). In addition to a function in open complex formation, TFIIH is able to repress the structure-specific endonuclease activities of both ERCC1-XPF and XPG (70). The inhibition is likely mediated by formation of ternary nucleoprotein complexes. Addition of ATP relieves inhibition by TFIIH suggesting that a conformational change involving ATP hydrolysis is required. Further evidence also implicates TFIIH in functions different from open complex

formation. A splice mutation resulting in alteration of the C-terminus of the XPB subunit of TFIIH is present in cells derived from a XP/CS patient (XP11BE). In extracts derived from these cells, full open complex formation is detected, but 5' incisions are absent (55). It is possible that the C-terminus of XPB is required for ATP-dependent conformational change resulting in relief of inhibition of the ERCC1-XPF nuclease, but not for relief of XPG inhibition.

Reversible phosphorylation of NER factors is important for dual incision activity *in vitro* (80). However, it is not clear which factors are targets for phosphorylation, nor which kinase is involved. RPA is a known incision factor that becomes hyperphosphorylated upon cellular UV-irradiation (81,82). However, the phosphorylation status of this protein does not affect its activity in the dual incision reaction, at least *in vitro* (80,83). TFIIH is the single factor involved in the incision stage that possesses a kinase activity. The role of the TFIIH-mediated kinase activity is not unambiguous: antibodies directed against the kinase catalytic subunit, CDK7, inhibit NER *in vivo* (14), but TFIIH lacking kinase activity is fully active in an *in vitro* reaction with highly defined components (52). The TFIIH-associated kinase is able to efficiently phosphorylate XPG *in vitro* (70). However, it is not evident what the physiological relevance is of this finding and whether phosphorylation of XPG is involved in the relief of inhibition by TFIIH. It may be speculated that the kinase activity is involved in, for example, recycling or turn-over of NER factors bound to reaction intermediates.

After dual incision, the damage-containing oligonucleotide is released from the remaining gapped-duplex DNA and not subject to exonucleolytic degradation by repair proteins (42,75). Because both DNA molecules are protein-bound in a system reconstituted with highly purified proteins (52), the release of the damage-containing oligonucleotide may only require dissociation of protein interactions, which may involve phosphorylation. With the release of the damage-containing fragment, the initial stage of NER is completed.

Gap-filling DNA repair synthesis

After dual incision and release of the DNA lesion as part of an oligonucleotide, the resulting gap is filled in by DNA replication factors. Repair DNA synthesis does not result in extensive repair patches caused by strand displacement, but is confined to a short region of about 30 nucleotides as measured by the incorporation of labeled or modified nucleotides *in vitro* (75,84,85) or *in vivo* (86) using a variety of techniques. Repair synthesis is strictly dependent on PCNA (87). Although DNA polymerase ε can complete gap filling efficiently in the absence of additional factors using deproteinized gapped substrates, the presence of RPA results in a strict dependence for replication factors RFC and PCNA. Ligation of the resulting nick can be carried out by DNA Ligase I. DNA polymerase δ can also carry out gap-filling synthesis. However, repair replication by DNA polymerase δ results in rather inefficient ligation. These data indicate that a combination of either DNA

polymerase ε or δ, PCNA, RPA, RFC, and DNA Ligase I is able to complete the formation repair patches *in vitro* (88).

Further evidence for the importance of both DNA polymerases in NER comes from yeast genetics. *S. cerevisiae* conditional double mutants for DNA polymerase δ or ε accumulate UV-induced single stranded breaks, suggesting that both DNA polymerases are physiologically important for the final stage of NER (89). However, single mutants do not accumulate single stranded gaps, indicating that these DNA polymerases can compensate for each other in yeast (89).

INITIATION OF RNA POLYMERASE II TRANSCRIPTION

NER is functionally linked with RNA polymerase II transcription. This is evident from the observation that certain lesions in actively transcribed genes are repaired faster than lesions present in inactive loci (37,90). Furthermore, TFIIH is required both in NER and RNA polymerase II transcription (4-8).

The transcription cycle can be divided into several closely coupled steps: (i) recruitment of transcription factors and RNA polymerase II to the promoter resulting in the formation of a pre-initiation complex; (ii) promoter opening, which is dependent on ATP hydrolysis; (iii) actual initiation, which is defined as the formation of the first phosphodiester bond; (iv) promoter escape, which denotes the transition from initiation to elongation; (v) elongation, the extension of the RNA transcript; and (vi) termination of transcription. In this section, the general initiation process corresponding to steps (i) to (iv) will be discussed in more detail with emphasis on the role of TFIIH (Figure 2).

Formation of a pre-initiation complex

Initiation of transcription requires basal transcription factors in addition to RNA polymerase II. The polymerase and the set of five basal transcription factors, TFIID, TFIIB, TFIIF, TFIIE, and TFIIH (Table 3), suffice for initiation of promoter-specific transcription *in vitro* (91). TFIID consists of TBP, which binds to the TATA-element, and a number of TBP-associated factors (TAF_{II}s). The TAF_{II} proteins are not essential for basal levels of transcription as TBP can replace the TFIID complex *in vitro* (reviewed in (92)). The set of basal factors may assemble onto a promoter as part of a pre-organized holo-enzyme, that contains most basal factors except TFIID (93,94), or by an ordered step-wise mechanism (95). The ordered assembly is nucleated by the binding of TFIID (TBP) to the TATA-element. This complex is subsequently recognized by TFIIB, which can bind to a small sequence located upstream of the the TATA box (96), TFIIF, which escorts RNA polymerase II to the pre-initiation complex, and finally TFIIE and TFIIH (reviewed in (91)). TFIIH is recruited into the pre-initiation complex by TFIIE (97),

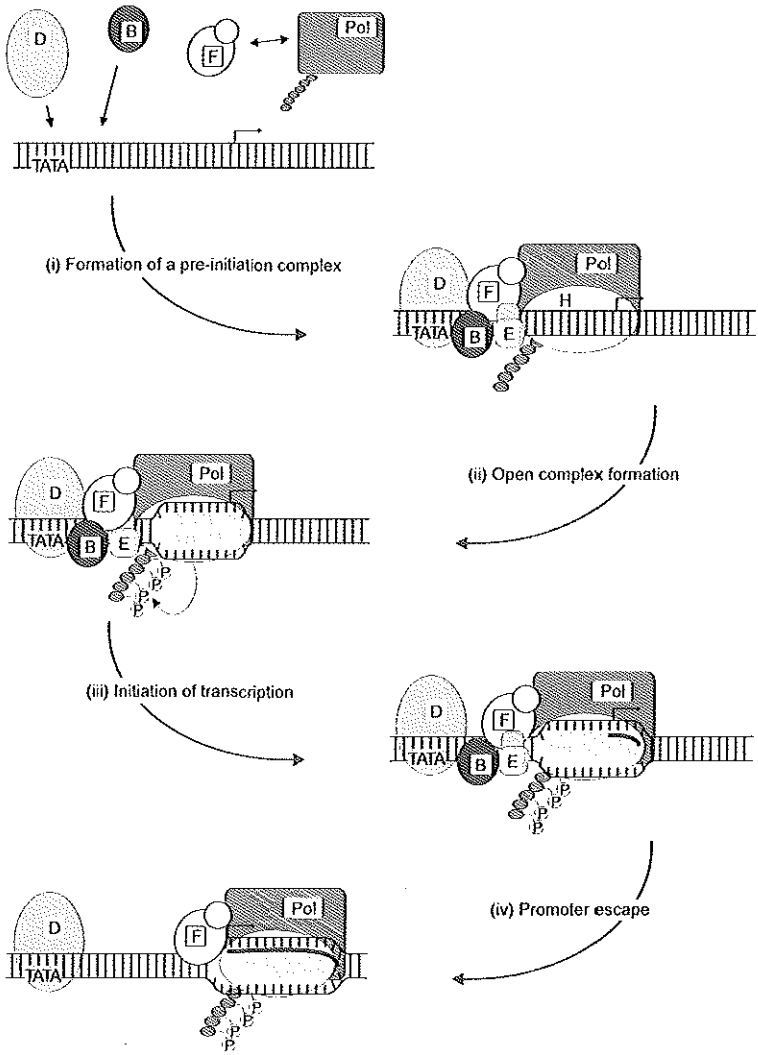

Figure 2. The transcription initiation cycle of RNA polymerase II. (i) Formation of a pre-initiation complex starts by binding of TFIID (TBP) to the TATA-element of the promoter of class II genes. Subsequently, TFIIB, and TFIIF and RNA polymerase II enter the complex. Under certain conditions, this set of protein factors is sufficient for promoter-specific transcription initiation. Pre-initiation complex formation is completed by entry of TFIIE and recruitment of TFIIH. (ii) Open complex formation. Addition of ATP activates the pre-initiation complex by utilizing the multiple enzymatic activities of TFIIH. A melted region encompassing the transcription start site and an upstream promoter part depends on the helicase activity of XPB, but not on the helicase activity of the XPD subunit of TFIIH. The C-terminal domain of the largest subunit of RNA polymerase II is hyperphosphorylated by the CDK7 kinase catalytic subunit of TFIIH. (iii) Actual initiation of transcription: the formation of the

first phosphodiester bond. Until the formation of a five-nucleotide nascent transcript, the RNA polymerase II requires continued ATP hydrolysis by TFIIH. (iv) Promoter escape: transition into a productive elongation complex. The fate of the initiation factors is schematically indicated: TFIIB and TFIIE are released before the formation of a 10-12 nucleotide transcript; TFIID remains promoter-bound and able to recruit TFIIB to the promoter; TFIIF and TFIIH remain associated with polymerase and are released during early stages of elongation.

which may depend on a direct interaction of the large subunit of TFIIE, p56, with the XPB subunit of TFIIH (7,98).

Table 3. Human basal transcription factors.

Factor	Subunit [a, b]	Properties	References
TFIIB	33	Binds core promoter-element	96
TFIID	250,	TBP-associated factors (TAF_{II}); $TAF_{II}250$ has kinase	154,155
	150, 130, 100, 55,	(phosphorylates RAP74) and histone acetyltransferase activity	
	31, 30, 28, 18	Histone-like TAF_{II}	
	80, 31, 20/15	Binds TATA-element	92,156
	38 (TBP)		
TFIIE	56 (α)		
	34 (β)		
TFIIF	74 (RAP74)	Phosphorylated by TFIID ($TAF_{II}250$)	154
	30 (RAP30)		
TFIIH	89 (XPB/ERCC3)	DNA-dependent ATPase, 3'-5' DNA helicase	11,12
	80 (XPD/ERCC2)	DNA-dependent ATPase, 5'-3' DNA helicase	11,12,145
	62		
	52		
	44	Zn-finger protein; putative DNA-binding	26
	34	Zn-finger protein	
	40 (CDK7)	Kinase catalytic subunit; subunit of CAK	14
	36 (Cyclin H)	subunit of CAK	
	32 (MAT1)	subunit of CAK	

[a] The numbers refer to the calculated molecular weight of the subunits (kD).
[b] References for cDNAs encoding these factors are reviewed elsewhere (27,91,92).

Open-complex formation: critical role for TFIIE and TFIIH

After assembly of the pre-initiation complex, hydrolysis of ATP is required to activate transcription by formation of an open complex. This involves melting of DNA strands around the transcription start site (99). The melting of a promoter region critically depends on TFIIE and TFIIH, and a β-γ hydrolyzable bond of ATP, which results in opening of ~12 bases mostly upstream of the transcription start site (100). The open complex is sensitive to ATPγS, indicating that it is unstable and requires continued ATP hydrolysis (100,101). Under some conditions, TFIIE and TFIIH are dispensable for transcription. It has been shown that heteroduplex regions around the initiation site of as little as 6 base pairs (from -4 to +2 relative to the initiation site +1) are sufficient to circumvent the requirements for ATP, TFIIE, and TFIIH (100,102,103). Negative superhelicity of promoter regions also alleviates the

need for these factors (104-106) probably by inducing transient melting of DNA, which may be localized to the promoter region in the absence of TFIIE and TFIIH. Together, these results firmly establish that TFIIH, utilizing its ATPase activity, melts a promoter region together with TFIIE, either by directly disrupting base pairing, or by imposing topological constraints onto the promoter DNA.

Open complex formation does not depend on both DNA-dependent ATPase activities associated with TFIIH. Purified TFIIH containing an active-site mutant XPD subunit supports basal transcription levels *in vitro* and promoter melting appears not affected by the presence of enzymatically inactive XPD (71,107). Furthermore, microinjection of a cDNA encoding active-site mutant XPD does not affect transcription *in vivo* in human fibroblasts (71), and yeast *rad3* cells carrying the corresponding inactivating mutation in the homolog of XPD are viable (108). In contrast, TFIIH with an active-site mutant XPB subunit does not support transcription both *in vitro* and *in vivo* (6,107). Also, yeast *rad25* strains carrying an active-site mutation in the XPB homolog are not viable (109). Together, these data indicate that XPD activity is dispensable for basal transcription and that promoter melting critically depends on the XPB-associated ATPase/DNA helicase activity.

The role of TFIIE in promoter opening is not solely to recruit TFIIH into the pre-initiation complex. Under conditions in which TFIIH is not required, TFIIE can influence the rate of transcription, depending on the helical stability of the promoter region just upstream the initiation site (103). Furthermore, TFIIE may regulate the enzymatic activities of TFIIH (7,110). The functional interplay between TFIIE and TFIIH is further demonstrated by studies with purified yeast factors. TFIIE obtained from *S. cerevisiae* cannot substitute for the corresponding factor in a system with purified *S. pombe* factors, unless both *S. cerevisiae* TFIIE and TFIIH together substitute their corresponding *S. pombe* factors (111).

Actual initiation and transition to elongation

After the formation of an open complex, the first phosphodiester bond is rapidly synthesized. The open region expands during extension of the RNA transcript, but remains sensitive to ATPγS until a four-nucleotide transcript is formed (112), which suggests an involvement of TFIIH in maintaining the open complex during the first stage of initiation. During the synthesis of the first 6-10 nucleotides of the nascent transcript, several rather undefined events take place. An active role for TFIIE and TFIIH involving hydrolysis of the β-γ bond of ATP by TFIIH appears to be required to prevent promoter proximal stalling and efficient promoter escape under conditions with limiting nucleotide concentrations (106,113,114).

A second important event required for the transition from initiation to elongation is the phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (reviewed in (115)). RNA polymerase II that is recruited to the pre-initiation complex is mainly unphosphorylated. An important CTD kinase is TFIIH, which phosphorylates the CTD efficiently in a highly purified

system (13) through the CDK7 catalytic subunit (14-16). Phosphorylation of RNA polymerase II by TFIIH is not required for basal transcription of all promoters: a potent CTD protein kinase inhibitor, the isoquinoline derivative H-8, does not significantly affect transcription from the adenovirus major late promoter *in vitro* (116). Furthermore, purified TFIIH with a deficient kinase subunit supports transcription *in vitro* from the same promoter (18). In contrast, the murine *DHFR* promoter requires the CTD, but not its phosphorylation, for initiation *in vitro*, while transcription strongly depends on CTD phosphorylation, probably during promoter escape (117).

During the transition to elongation, and before the synthesis of a 10-nucleotide transcript, most basal factors have dissociated from transcribing RNA polymerase II (TFIIB, TFIIE), or remain bound to the promoter (TFIID/TBP) in a highly defined system. Under these conditions, TFIIH is released from RNA polymerase II from early elongating complexes after synthesis of transcripts of 30-68 nucleotides (118).

TRANSCRIPT ELONGATION AND THE EFFECT OF DNA DAMAGE

Elongation of transcription by RNA polymerase II is characterized by non-uniform processivity of the polymerase through a gene. Discrete sites of pausing, and premature arrest may occur as a result of a specific nucleotide context or by proteins bound to DNA (119). Several factors have been implicated in regulation of elongation, including TFIIH. Microinjection of antibodies directed against TFIIH subunits can block transcript elongation in *Xenopus* oocytes, which is not observed with antibodies directed against the initiation specific factor TFIIB (120). Furthermore, a known inhibitor of elongation, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB), also inhibits the TFIIH-associated CTD kinase, suggesting an involvement of the kinase activity of TFIIH in this process (121). Although RNA polymerase II does not require a DNA helicase for processivity *in vitro*, it is presently not clear whether the helicase subunits of TFIIH are also involved in transcript elongation.

Several types of DNA lesions can affect elongation by RNA polymerase II. In transcription systems with purified components, a CPD photoproduct is a potent block to transcribing RNA polymerase II *in vitro* (122). For this effect, the CPD lesion has to be present in the transcribed strand. The stalled RNA polymerase II at a CPD lesion, protects the DNA around the damage as determined by footprinting and resistance to repair by the *A. nidulans* photoreactivating enzyme (122). The bulky aromatic AAF adducts located on the transcribed strand also cause transcription arrest at lesion sites, which is not seen with the less bulky 2-aminofluorene (AF) adducts (123). Stalling of RNA polymerase II at sequence-determined or damage-containing sites may induce recruitment of factors that stimulate transcription readthrough. Transcription elongation factor TFIIS facilitates by-pass of certain transcription pause sites. Upon pausing at a particular site,

movement of the RNA polymerase II complex may be induced in the backwards direction instead of forming the next phosphodiester bond. To realign the RNA-DNA hybrid with the catalytic site, 3' end cleavage of the nascent transcript is induced by TFIIS (see (119) and references therein). The effect of TFIIS on pausing caused by DNA lesions has been investigated. TFIIS induces upstream movement and transcript cleavage by RNA polymerase II paused at a CPD lesion (122), but not at an AAF adduct site located in the transcribed strand *in vitro* (123).

TRANSCRIPTION-COUPLED REPAIR

Certain lesions present in actively transcribed genes are repaired at a faster rate compared to those present in inactive loci (36,37). Moreover, the increased repair rate of active genes is mainly due to the preferential repair of the transcribed strand (90). This phenomenon, which is referred to as transcription-coupled repair, depends on the CSB and CSA proteins, and is independent of the XPC-HR23B complex.

The mechanism of transcription-coupled repair is not well understood. The SWI/SNF family member CSB contains DNA-dependent ATPase activity (124,125) and is associated *in vivo* with a RNA polymerase II complex (126). As complexes containing RNA polymerase II and CSB do not contain the general initiation factors, it was suggested that CSB associates with a fraction of elongating RNA polymerase II (126). The association of purified CSB with RNA polymerase II depends on transcription, and possibly on stalling at either a defined pause-sequence, oxidative base damage, or a double-stranded DNA end, and is dependent on hydrolyzable ATP (127). Complexes consisting of CSB, RNA polymerase II, and a DNA-RNA hybrid are able to recruit TFIIH (128). Although direct interactions have been reported between CSB and TFIIH subunits (124,129), it is most likely that the recruitment of TFIIH involves additional factors (128). If stalling of the polymerase at the lesion and subsequent association of proteins including CSB and TFIIH represent the first stages of transcription-coupled repair, then at least three scenarios can be envisioned for the following events (Figure 3). First, these proteins may induce upstream movement of the polymerase, possibly involving the action of TFIIS to realign the DNA-RNA hybrid. This would allow removal of the lesion by the NER machinery. After removal of the damage, transcription may resume (130). Second, the complex including stalled RNA polymerase II, CSB and TFIIH may favour transcriptional by-pass of the lesion and continuation of transcription before repair of the damage. After resumption of transcription, fast repair of the injury may prevent accumulation of transcripts containing specific mutations. The increased repair-rate may be achieved by removal of a rate-limiting step from the NER reaction. These may include: (i) the relatively large size of the transcription bubble may result in an increased damage recognition rate in an XPC-HR23B independent manner due to the presence of the lesion in a locally unwound conformation (53); (ii) a high local concentration of

TFIIH may facilitate recruitment of this factor possibly resulting in faster repair; (iii) the upstream transcription bubble may partly bypass the helix-unwinding step also resulting in higher repair rates. A third possible model would include displacement of stalled RNA polymerase II from the DNA allowing access of the NER machinery to the damaged site and removal of the lesion. However, in this model it is not easily explained how XPC-HR23B independent recognition is obtained. In addition, neither recombinant CSB nor CSA appears to be able to remove RNA polymerase II from DNA (124).

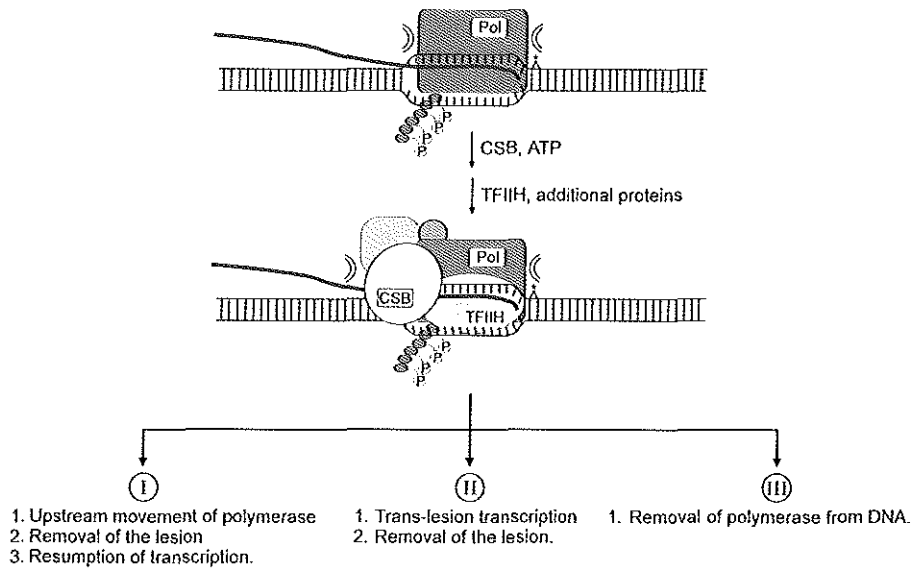

Figure 3. Possible models for transcription-coupled NER. RNA polymerase II can be blocked by certain DNA lesions located in the transcribed strand. The CSB protein may be able to associate with a stalled polymerase in an ATP-dependent fashion. A complex of CSB and RNA polymerase II is subsequently able to recruit TFIIH and possibly additional (NER) factors, which may result in: I. Removal of the lesion, before transcription resumes; or: II. Stimulation of lesion by-pass by RNA polymerase II, thereby making the lesion available for repair; or: III. Removal of the polymerase from the DNA permitting repair of the damage.

Interestingly, transcription-coupled NER of DNA damage positioned within approximately 50 nucleotides from a transcription start site does not require the RAD26 protein, the homolog of CSB, in yeast cells (131). Possibly, the fast repair in this region is due to the association of TFIIH with RNA polymerase II. Similarly, transcription-coupled NER of DNA lesions around the transcription initiation site is not deficient in human CS-B cells (132).

Recruitment of TFIIH *via* CSB and other proteins may represent a more general mechanism by which elongation rates of paused polymerase are stimulated. Indeed,

like is the case for TFIIH, several lines of evidence have implicated CSB with a role in elongation (133,134). Based on the patient condition and cellular phenotype, it is likely that, in addition to CSB, the CSA protein cooperates in the same mechanism. However, the biochemical properties of CSA need to be characterized in more detail to identify the role of CSA in the transcription-coupled repair pathway of NER.

REGULATORY FACTORS OF TFIIH ACTIVITY

TFIIH is subject to regulation via post-translational modifications. Not much is known, however, about the mechanisms or factors involved. Only few examples are rather well described, and these will be discussed below.

S. cerevisiae mms19 strains are UV-sensitive and display a temperature-sensitive growth and methionine auxotrophy (135). The first two phenotypic characteristics are very similar to those of *rad25* and *rad3* strains, which have mutations in the yeast homologs of the human *XPB* and *XPD* genes, respectively, and suggest an involvement for MMS19 in NER and RNA polymerase II transcription. The temperature-sensitive transcription defect in *mms19* extracts can be complemented by addition of highly purified TFIIH, although the MMS19 protein is not a subunit of TFIIH (135). Likewise, the NER deficiency in *mms19* extracts cannot be complemented by addition of *rad3* extracts, although extracts with a defect in the XPA homolog, Rad14, can, supporting the idea that the defect in NER is due to a defective function of TFIIH (136). As MMS19 is not required for both transcription and NER in highly purified systems, these data suggest that the protein is involved in the regulation of TFIIH activity. It also indicates that TFIIH can complement extracts with NER or transcription deficiencies that are not necessarily caused by a mutation in a TFIIH subunit. The latter finding may be of importance to understand the complementation of the NER defect in TTD-A cells by microinjection of highly purified TFIIH (2,137). Because the primary defect appears not to be caused by a mutation in a gene encoding any of the TFIIH subunits, the defect in TTD-A may be caused by mutational inactivation of a factor involved in the regulation of TFIIH. A human homolog of *S. cerevisiae* MMS19 was identified and its cDNA cloned (138). The protein is not defective in TTD-A as no mutations could be identified in the cDNA derived from the patient cell line. To corroborate the involvement of human MMS19 in NER and its relation with TFIIH, it may be of interest to generate a mammalian mutant by inactivation of the murine *MMS19* gene by homologous recombination.

The transcriptional activity of TFIIH appears to be a target for regulation by some components that control progression through the cell cycle. Entry into mitosis is accompanied by repression of transcription by inactivation of key components of the transcriptional machinery (139). RNA polymerase II transcription is specifically inactivated in mitotic cells by inactivation of TFIIH and its associated kinase activity (140,141). The mitotic kinase cdc2/cyclin B is able to inhibit transcription in a purified transcription system, which can be reversed by the cyclin-dependent

kinase inhibitor p21/Waf1/Cip1 (140). Together, this suggests that repression is due to phosphorylation of TFIIH subunits. Indeed, a critical serine residue at position 164 in the CDK7 kinase catalytic subunit of TFIIH is phosphorylated in extracts from mitotic cells, which results in inhibition of the TFIIH-associated transcription and CTD kinase activities (141). Another protein involved in cell cycle regulation and implicated with regulation of TFIIH transcriptional activity is the cyclin-dependent kinase inhibitor p16/Ink4A (142). The recombinant protein binds to both the XPB and CDK7 subunits of TFIIH *in vitro* as well as to the CTD of the largest subunit of RNA polymerase II thereby inhibiting phosphorylation of the CTD and transcription (142). It is unknown what the effect is of the cell cycle dependent phosphorylation of CDK7 on the activity of TFIIH in NER. Furthermore, it will be of interest to find out whether and how the activities of TFIIH are regulated by cell cycle arrest induced by DNA damaging agents.

TRANSCRIPTION/DNA REPAIR SYNDROMES

Mutations in genes encoding TFIIH subunits have sofar only been found in the two genes encoding the XPB and XPD helicases. Defects in both subunits are associated with three distinct genetic disorders: xeroderma pigmentosum (XP), Cockayne's syndrome (CS), combined XP/CS, and a photosensitive form of trichothiodystrophy (TTD) (35). A summary of clinical symptoms associated with these syndromes is presented in Table 4. All three syndromes have in common (extreme) sun (UV) sensitivity. This and other characteristics for XP, sun-induced pigmentation abnormalities and a >2000-fold increased chance for skin cancer, can be rationalised on the basis of a NER defect. Similar symptoms can result from defects in other NER genes. However, many mutations in the genes encoding the TFIIH helicase subunits give rise to additional symptoms associated with CS or TTD: severely impaired physical, neurological and sexual development as well as prominent premature ageing and -in the case of TTD- additionally the characteristic brittle hair and nails and scaly skin. Given the dual involvement of TFIIH in NER and transcription, it has been proposed that symptoms associated with XP are due to an impairment in NER, while clinical characteristics associated with CS or TTD are caused by a transcription deficiency (137). The best documented role of TFIIH in transcription is in the initiation phase and, therefore, typical CS and TTD features as observed in some patients in the XP-B and XP-D complementation groups, are ascribed to different defects in TFIIH function in the transcription initiation stage (137,143). However, it may also be speculated that typical CS features are due to a defect during the elongation stage in a mechanism that may involve CSA, CSB, and TFIIH (128). The characteristic brittle hair and nails seen in TTD patients belonging to the XP-B and XP-D complementation groups may be due to additional defects in TFIIH, such as diminished activity in transcription initiation or reduced *in vivo* stability of the protein complex (see for discussion of the latter (143) and references therein).

Table 4. Clinical features of XP, XP/CS, CS, and TTD patients.

Feature	XP [a]	XP/CS	CS	TTD
Cutaneous				
Photosensitivity	++	++	+	+
Pigmentation ababnormalities	++	+	–	–
Brittle hair/nails	–	–	–	+
Ichtyosis	–	–	–	+
Skin cancer	++	+	–	–
Neurologic				
Primary defect:				
neurodegeneration	+	–	–	–
neurodysmyelination	–	+	+	+
Progressive metal degeneration	–/+	+	+	+
Wizened facies	–	+	+	+
Developmental				
Growth defects	–	+	+	+
Immature sexual development	–	+	+	+
Cellular				
UDS (% of control)	5–50%	5–50%	normal	5–50%
UV–sensitivity	++	++	+	+ / ±

[a] Does not include the XP-Variant form.

Only three mutant alleles (in a total of five patients) have been identified in XPB, compared to over 20 in XPD. The striking difference in these numbers can be rationalized by the helicase requirements for TFIIH function in transcription: whereas XPD helicase activity is dispensable, XPB activity is essential (6,71,107). Thus, the window for non-lethal mutations in XPB may be much smaller compared to XPD. The notion that transcription critically depends on XPB activity is also reflected in the condition of patients carrying mutations in this gene. All three mutations give rise to combined XP/CS or TTD. In contrast, mutations in XPD can give rise to XP, which may be due to disruption of the helicase activity in the absence of significant changes in the protein structure. Mutations resulting in additional conformational alterations of XPD may give rise to combined XP/CS or TTD.

CONCLUDING REMARKS

The biochemical mechanisms of NER and RNA polymerase II transcription initiation are understood in considerable detail. Both processes have in common an obligatory step involving local DNA melting. The enzymatic activities associated with TFIIH implicate this complex with a direct function during this step. However, the requirements for TFIIH in both DNA repair and transcription are not restricted to DNA unwinding. Many questions remain as to what the precise function is of TFIIH in transcription elongation and what the implications are for transcription-coupled repair. It will also be of interest to find out which proteins have the ability

to regulate TFIIH activity, such as cell cycle control factors, proteins similar to MMS19, and perhaps the factor defective in TTD-A cells. Finally, the understanding of how mutations in the helicase subunits affect the function of the complex during the multiple stages of both NER and transcription will ultimately provide a biochemical basis for human syndromes associated with a defective function of TFIIH. No doubt, the analysis of TFIIH will be a topic of intense and fruitful research for years to come.

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CHAPTER 2

Affinity Purification of Human DNA Repair/Transcription Factor TFIIH Using Epitope-tagged Xeroderma Pigmentosum B Protein

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Affinity Purification of Human DNA Repair/Transcription Factor TFIIH Using Epitope-tagged Xeroderma Pigmentosum B Protein*

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TFIIH is a high molecular weight complex with a remarkable dual function in nucleotide excision repair and initiation of RNA polymerase II transcription. Mutations in the largest subunits, the XPB and XPD helicases, are associated with three inherited disorders: xeroderma pigmentosum, Cockayne's syndrome, and trichothiodystrophy. To facilitate the purification and biochemical characterization of this intricate complex, we generated a cell line stably expressing tagged XPB, allowing the immunopurification of the XPB protein and associated factors. Addition of two tags, a N-terminal hexameric histidine stretch and a C-terminal hemagglutinin epitope, to this highly conserved protein did not interfere with its functioning in repair and transcription. The hemagglutinin epitope allowed efficient TFIIH immunopurification to homogeneity from a fractionated whole cell extract in essentially one step. We conclude that the predominant active form of TFIIH is composed of nine subunits and that there is one molecule of XPB per TFIIH complex. The affinity-purified complex exhibits all expected TFIIH activities: DNA-dependent ATPase, helicase, C-terminal domain kinase, and participation in *in vitro* and *in vivo* nucleotide excision repair and *in vitro* transcription. The affinity purification procedure described here is fast and simple, does not require extensive chromatographic procedures, and yields highly purified, active TFIIH.

Nucleotide excision repair (NER)[1] is a versatile DNA repair mechanism that removes a wide variety of lesions, such as UV-induced lesions and numerous chemical adducts (1, 2). The principal steps in the reaction mechanism of NER are recognition and demarcation of the lesion, probably involving chromatin remodelling and local helix opening, incision of the DNA on both sides of the lesion at some distance, removal of the damaged oligonucleotide, and, finally, repair DNA synthesis and ligation. In eukaryotes, this reaction requires about 30 polypeptides and has been reconstituted with purified components, including XPA, XPC/HHR23B, replication protein A, the structure-specific nucleases ERCC1/XPF and XPG, and the multisubunit basal transcription factor TFIIH (3–5). At least two subpathways can be discerned in the NER system. One of these, transcription-coupled repair, preferentially removes DNA damage from the transcribed strand of active genes, whereas lesions in the rest of the genome are repaired more slowly and less efficiently by the global genome repair pathway. TFIIH appears to be a core component of both excision subpathways.

Mutations in the two largest subunits of TFIIH, the XPB and XPD helicases, are associated with the rare genetically heterogeneous disorders xeroderma pigmentosum (XP), Cockayne's syndrome (CS), and trichothiodystrophy (TTD) (6, 7). Many complementation groups and considerable overlap have been established for these syndromes: seven complementation groups in XP (XP-A–XP-G), three of which include patients with combined XP and CS phenotypes (XP-B, XP-D and XP-G), two in the classical form of CS (CS-A and CS-B), and three in TTD (XP-B, XP-D, and TTD-A). The discovery of the dual function of XPB and XPD in both NER and transcription provides a rationale for the complex clinical features that are specifically associated with inherited defects in TFIIH subunits, such as seen in the combined XP/CS and the photosensitive form of TTD, that were difficult to explain solely on the basis of a NER defect. Thus, it was proposed that typical XP characteristics, such as UV-induced cutaneous abnormalities and predisposition to skin cancer, are due to inactivation of the NER function of TFIIH, whereas features typical for CS and/or TTD, such as neurodysmyelination, brittle hair, and growth defects, are due to a deficiency in the transcription function of TFIIH, possibly affecting only a subset of genes (8).

In addition to XPB and XPD, which exhibit DNA-dependent ATPase activities and are 3′-5′ and 5′-3′ DNA helicases, respectively (9, 10), seven more TFIIH subunits have been identified to date. CDK7 was identified as the catalytic subunit of the kinase activity of TFIIH that is able to phosphorylate the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (11). Interestingly, CDK7 also constitutes a separate trimeric kinase complex that is possibly involved in cell cycle regulation together with the cyclin H and MAT1 subunits of TFIIH (12, 13). Furthermore, p44, the human homologue of yeast SSL1, and p34 contain zinc finger domains and possess putative DNA binding capacity (14). So far, no activity has been detected for the p62 and p52 subunits (15, 16). Whether these nine proteins constitute the TFIIH complex and whether the composition of TFIIH differs during NER and transcription initiation are yet unresolved issues.

The presence of two DNA helicases has implicated TFIIH in

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[1] The abbreviations used are: NER, nucleotide excision repair; XP, xeroderma pigmentosum; CS, Cockayne's syndrome; TTD, trichothiodystrophy; CTD, C-terminal domain; WCE, whole cell extract; HA, hemagglutinin; dtXPB, double-tagged XPB.

a helix-opening step during both transcription initiation and NER. It has been shown that such open-complex formation at the transcription start site depends on TFIIH and that the requirement for TFIIH is dependent on promoter topology and can be alleviated by premelted regions at the transcription start site (17–19). During NER, TFIIH is thought to convert a recognized damaged site into a substrate for the XPG and XPF/ERCC1 structure-specific nucleases by locally opening DNA around a lesion. The formation of an opened DNA conformation around a recognized lesion has been demonstrated; however, the direct involvement of TFIIH in this step has not been shown (20). Answers to these questions are hampered by the difficulty in obtaining large quantities of highly purified TFIIH. Therefore, we developed a procedure that facilitates the isolation of TFIIH using a human cell line expressing functional XPB provided with two tags. The affinity purification procedure described here is fast and simple, does not require extensive chromatographic procedures, and yields highly purified, active TFIIH.

EXPERIMENTAL PROCEDURES

General—Purification of nucleic acids, restriction enzyme digestion, gel electrophoresis of nucleic acids and proteins, immunoblotting, detection of proteins and nucleic acids were performed according to standard procedures (21).

Oligonucleotides and Plasmid DNA Construction—The coding sequence for the C-terminal HA epitope tag was added via PCR using oligonucleotide primer pairs p90 5′-CCCGGATCCTCAGCTAGCGTAATCTGGAACATCGTATGGGTATTTCCTAAAGCGCTTGAAG-3′ (3′ primer; underlined sequence encodes the HA epitope, and double underlined sequence indicates a *Bam*HI restriction site) and p33 5′-GGATCCACCATGGGCAAAAGAGACCG-3′ (5′ primer). Likewise, the hexahistidine tag was added using oligonucleotides p123 5′-CGCGCGGAATTCATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGGCAAAAGAGACCG-3′ (5′ primer; underlined sequence encodes a hexahistidine stretch and thrombin cleavage site, and double underlined sequence indicates an *Eco*RI site) and p41 5′-CGGGAAGTGGAGGGCCCACC-3′ (3′ primer). PCR fragments were cloned, sequenced, and confirmed to be free of PCR-introduced sequence errors. Full-length *XPB* and double-tagged *XPB* cDNA (*dtXPB* cDNA) were subcloned as *Eco*RI-*Bam*HI fragments in a modified pSG5 eukaryotic expression vector yielding plasmids pSHE3 and pSHE3HA, respectively. From pSHE3HA, the *dtXPB* cDNA was subcloned as a *Eco*RI-*Xho*I fragment in the eukaryotic expression vector pcDNA3 (Invitrogen) yielding plasmid pM300.

DNA Transfection and UV Survival Assay—Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-10 medium supplemented with 10% fetal calf serum and antibiotics. HeLa TK^- cells were transfected with vector pM300 by electroporation, and after selection with 1.0 mg/ml G418, individual clones were selected for expression of *dtXPB* by immunoblot analysis using anti-XPB antibodies.

Cell lines XP-wt and XP-t3 were generated by transfection of XPCS2BASV (XP-B) SV40-immortalized fibroblasts with *XPB* and *dtXPB* cDNA, respectively. Expression vector pSHE3 or pSHE3HA and a *neomycin*-selectable marker, were cotransfected using Lipofectin reagent essentially as described by the manufacturer (Life Technologies, Inc.). Stably expressing mass populations were obtained by selection with 300 μg/ml G418 and repeated UV irradiation at 1 day intervals (three doses of 4 J/m^2).

UV survival was assayed by [*methyl*-^{3}H]thymidine incorporation 3 days after irradiation as described (22). Proliferating cells were pulse-labeled with [*methyl*-^{3}H]thymidine (3 h) and then chased (1 h). Cell lysates were transferred to scintillation-counting vials, and survival was calculated as the average ratio of incorporated radiolabel in treated triplicates to that in four untreated control dishes.

Immunopurification of dtXPB Protein and Associated Factors—XP-t3 cells were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. Whole cell extracts (WCEs) (total, 530 mg) were prepared from frozen cell pellets (total, 50 ml packed cell volume) as described (23). Subsequently, WCEs were fractionated on heparin-Ultrogel (IBF, France) equilibrated in Buffer A (10 mM Tris-HCl, pH 7.8, 17% glycerol (v/v), 0.5 mM dithiothreitol, 5 mM $MgCl_2$) containing 0.1 M KCl and eluted with Buffer A containing 0.22, 0.4, and 1.0 M KCl as described (24). All of the dtXPB protein was present in the 0.4 M KCl fraction (designated Hep0.4; 175 mg of protein) as judged by immunoblot analysis similar to TFIIH. Typically, 10–12 ml (7.5–9.0 mg of protein) of the Hep0.4 fraction was successively incubated with 400 μg of purified 12CA5 anti-HA monoclonal antibody bound to 400 μl of protein G-Sepharose (Pharmacia Biotech Inc.) 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 dithiothreitol, 5 mM $MgCl_2$) containing 0.4 M KCl and 0.1% Nonidet P-40 and twice with buffer T/0.1 M KCl containing 0.01% Nonidet P-40. Bound material was eluted for 1 h at 30 °C in 400 μl of buffer T/0.1 M KCl containing 0.01% Nonidet P-40, 0.2 mg/ml insulin, 2.0 mg/ml synthetic peptide corresponding to the HA epitope (sequence YPYDVPDYA), and 1.0 μg/ml aprotinin. This step was repeated once or twice. Routinely, nearly all dtXPB and associated factors were present in the first eluate as detected by immunoblotting. Purified proteins were stored at −80 °C.

The same procedure was used for immunoprecipitation of dtXPB directly from a WCE (15 mg) to analyze protein-protein interactions. However, in this case, bound material was washed five times with buffer containing 0.1 M KCl, 0.1% Nonidet P-40 and analyzed by immunoblotting after overnight elution at 4 °C with buffer containing 1.0 mg/ml peptide and 0.1 mg/ml bovine serum albumin (Sigma).

Microneedle Injection—Prior to microinjection, human primary fibroblasts were fused by inactivated Sendai virus as described earlier (8). Protein fractions were microinjected into the cytoplasm of XP polykaryons, and NER activity was measured by pulse labeling with [*methyl*-^{3}H]thymidine and *in situ* autoradiography as described (8). Repair activity was quantified by counting autoradiographic grains above at least 50 non-S phase nuclei. Primary cell lines used were XPCS1BA (XP-B), XP6BE (XP-D), XP126LO (XP-F), XP3BR (XP-G), TTD1BR (TTD-A), and C5RO (wild type).

In Vitro NER Assay—WCEs were prepared from repair-proficient HeLa and XP-t3 cells and repair-deficient SV40-immortalized XPCS2BASV (XP-B) cells (23). NER reactions (50 μl) contained 250 ng of plasmid DNA randomly damaged with *N*-acetoxy-2-acetylaminofluorene and as an internal control an equal amount of undamaged control plasmid of a different size, the indicated amount of extract, and purified proteins; the reactions were incubated for 3 h at 30 °C. DNA was purified, linearized with *Bam*HI, and analyzed on a 0.8% agarose gel (25, 26). Antibody depletion of extracts was performed as follows: for each reaction, 100 μg of WCE was incubated with 0.5 μl of anti-p62 ascites and 5 μl of protein G-agarose overnight at 4 °C. For the experiment shown in Fig. 6*A*, plasmid DNA was randomly damaged with *cis*-diaminedichloro-platinum(II) (16).

In Vitro Transcription Assay—Purified TFIIH was incubated with recombinant human TBP, TFIIB, and TFIIE and purified TFIIA, TFIIF, and RNA polymerase II as described earlier (24). After 15 min of preincubation at 25 °C with 70 ng of linearized template DNA containing the adenovirus 2 major late promoter, nucleotides were added, and transcription was allowed to proceed in a final reaction volume of 25 μl for 45 min at 25 °C. The 309-nucleotide runoff transcripts were analyzed by electrophoresis through a 5% acrylamide/50% urea gel.

Enzymatic Assays—ATPase reactions contained 20 mM Tris-HCl, pH 7.9, 4 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 150 ng of DNA and were performed essentially as described before (27). After 30 min of incubation at 37 °C, 25-μl reactions were stopped by adding 2 μl of 0.5 M EDTA and 25 μl of TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Of each reaction, 1 μl was analyzed by thin-layer chromatography using polyethylenimine-cellulose plates (Merck) run in 0.75 M KH_2PO_4. CTD kinase assays (20 μl) containing 20 mM Hepes-KOH, pH 7.9, 20 mM Tris-HCl, pH 7.9, 7 mM $MgCl_2$, 0.5 μg/ml bovine serum albumin, and 30 mM KCl were performed with 10 μg of a synthetic tetrapeptide of YSPTSPS as a substrate as detailed before (27). DNA helicase probes were prepared as described (27), and reactions (25 μl) contained 20 mM Tris-HCl, pH 7.9, 4 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 2 mM ATP, and 1 ng of DNA substrate and were incubated at 37 °C for 45 min. Displacement of the 24-mer oligonucleotide from M13mp18 single-stranded DNA was analyzed by 10% nondenaturing polyacrylamide gel electrophoresis and autoradiography (27).

Antibodies—The monoclonal antibodies recognizing TFIIH subunits were all described before (10, 12, 14–16). Monoclonal antibodies recognizing the HA epitope (28) were purified from 12CA5 hybridoma tissue culture supernatant by affinity chromatography on protein G-agarose according to established protocols (29).

RESULTS

Generation of a Cell Line Expressing Tagged XPB—To analyze interactions of the XPB protein with other proteins, including TFIIH under physiological conditions, and to facilitate the purification of active TFIIH, we decided to generate a human cell line stably expressing a tagged version of *XPB* (*dtXPB*) cDNA. To permit isolation of full-length XPB protein and allow purification of XPB on the basis of different reversible affinity purification steps, we chose to add two different types of tags, one on each end of the protein. Thus, coding sequences for a N-terminal hexahistidine stretch followed by a thrombin cleavage site and a C-terminal HA epitope tag (28, 30) were added to *XPB* cDNA fragments, and a full-length double-tagged *XPB* cDNA was constructed (Fig. 1*A*) and subcloned in eukaryotic expression vectors. To obtain cell lines stably expressing *dtXPB*, the cDNA vectors were transfected to two human cell lines. First, *dtXPB* cDNA and a *neomycin*-selectable marker were transfected into HeLa cells. After selection with G418, individual HeLa clones were obtained and analyzed by immunoblotting for the level of the dtXPB protein using anti-XPB antibodies. As shown in Fig. 1*B*, *lanes 1–5*, the double-tagged XPB can be conveniently discerned from the endogenous wild type XPB protein because of its increased size (the predicted molecular mass increases from 89,279 Da to 92,690 Da). In the 48 clones analyzed, various levels of dtXPB protein were detected in WCEs, with many clones expressing no or hardly detectable *dtXPB*, despite the fact that the *dtXPB* cDNA was under the control of the strong cytomegalovirus promoter, and multiple copies are expected to be integrated in the genome. Interestingly, in neither case did we observe a large overexpression, and clones expressing the highest level of *dtXPB* clearly showed decreased levels of the wild type endogenous protein as compared with the p62 core subunit of TFIIH (*e.g.* Fig. 1*B*, compare *clone 1-19* with *clones 1-6, 1-2*, and *1-7*). These findings suggest that the cellular content of XPB is kept within narrow concentration ranges by degrading excess protein and that there is competition between the endogenous wild type and exogenous tagged protein. Secondly, XP-B UV-sensitive cells were transfected, and after selection with G418 and repeated UV irradiation, a stably expressing mass population was established, designated XP-t3. As with the HeLa transfectants, immunoblot analysis of XP-t3 cell extracts indicated that dtXPB protein levels were comparable with the endogenous (mutant) XPB levels (Fig. 1*B*, *lanes 6–8*). Because the relatively high dtXPB protein levels in HeLa clone 1-19 varied during culturing and appeared to be more stable in the transfected XP-B cells, the XP-t3 cell line was further characterized and used for all experiments described here.

To characterize the functionality of the dtXPB protein *in vivo*, an UV survival experiment was carried out. Fig. 1*C* shows that *dtXPB* is able to reverse the UV sensitivity of XP-B cells to the same extent as wild type *XPB* cDNA, although both not to the same level as the wild type MRC5 transformed cell line used as a repair-proficient control. The NER activity was further analyzed using an *in vitro* assay. WCEs were prepared and incubated in the presence of both damaged and undamaged plasmid DNA and labeled nucleotides. The resulting incorporation of labeled nucleotides in the damaged plasmid DNA is due to repair DNA synthesis and a measure for NER activity. Fig. 1*D* shows that extracts prepared from XP-t3 cells were able to repair *N*-acetoxy-2-acetylaminofluorene-damaged DNA *in vitro*, whereas extracts prepared from XP-B cells display a strongly reduced repair activity. The above findings indicate that the dtXPB protein is functional in NER, implying that it most likely is incorporated in the TFIIH complex. Finally, the fact that these cells grow normally strongly suggests that the dtXPB protein is not interfering with the basal transcription initiation function of the complex.

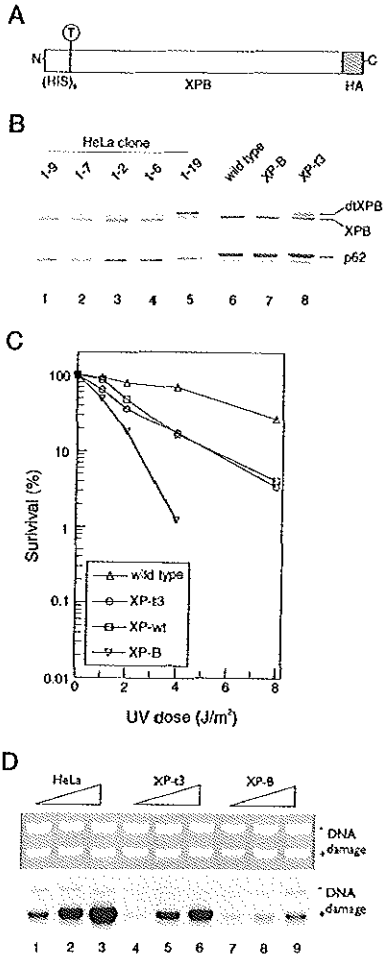


FIG. 1. **Analysis of the XP-t3 cell line expressing *dtXPB*.** *A*, schematic representation of the dtXPB protein. Indicated are the hexahistidine stretch (*$(HIS)_6$*), the thrombin cleavage site (*T*), and the HA epitope tag (*HA*). *B*, immunoblot analysis of cloned HeLa transfectants and the XP-t3 cell line containing varying levels of dtXPB protein. Due to the increased size of the double-tagged XPB, the protein migrates at a higher molecular mass. *Lanes 1–5*, HeLa clones containing varying levels of dtXPB protein; *lane 6*, wild type immortalized fibroblast (VH10); *lane 7*, XP-B immortalized fibroblast (XPCS2BASV); *lane 8*, XP-t3 transfected XP-B (XPCS2BASV) fibroblast. Indicated are the endogenous and tagged XPB proteins and the p62 subunit, used as internal reference for the amount of TFIIH in each lane. The p62 subunit appears in *lanes 6–8* as a doublet due to posttranslational modifications (T. Seroz, J. Auriol, and J.-M. Egly, unpublished results). *C*, dtXPB is functional in DNA repair. UV survival of XPCS2BASV (XP-B) fibroblasts and transfectants expressing tagged and wild type *XPB* cDNA (*XP-t3* and *XP-wt*, respectively). As a wild type control, MRC5 immortalized fibroblasts were used. *D*, *in vitro* NER activity measured in WCEs from HeLa (*lanes 1–3*), XP-t3 (*lanes 4–6*), and XP-B (*lanes 7–9*) cells using *N*-acetoxy-2-acetylaminofluorene-damaged plasmid DNA as substrate. Of each WCE, 50, 100, and 150 μg was used, respectively. *Upper panel*, ethidium bromide-stained gel of the linearized damaged and undamaged plasmids; *lower panel*, corresponding autoradiogram.

Analysis of TFIIH Factors Associated with dtXPB Pro-

tein—To identify proteins interacting with XPB, an immunoprecipitation experiment was carried out under physiological salt conditions (0.1 M KCl) using a repair- and transcription-competent XP-t3 WCE. As shown in Fig. 2, the dtXPB protein could be specifically and quantitatively immunoprecipitated using anti-HA monoclonal antibodies, and in addition to dtXPB, we could detect several TFIIH subunits in the bound fraction (XPD, p62, CDK7, and cyclin H). This confirms that dtXPB was incorporated in TFIIH. Furthermore, the fact that the relative intensities of XPB *versus* p62 are not significantly altered in the load, unbound, and the bound fractions indicates that the established XP-t3 cells harbor dtXPB in the majority of the TFIIH complexes. Interestingly, although all dtXPB protein was depleted from the WCE, none of the endogenous (mutant) XPB was detected in the bound fraction. This demonstrates that only one XPB subunit is present per complex: if the complex contained more than one XPB molecule, complexes with both the endogenous (mutant) subunit and the tagged protein would be expected to be present in the bound material.

Immunopurification of XPB and Associated Factors—Because a number of TFIIH subunits specifically co-immunoprecipitated with dtXPB protein using anti-HA antibodies, we set out to purify TFIIH on this basis and analyze the composition of the TFIIH complex (Fig. 3*A*). First, WCEs from XP-t3 cells were fractionated on heparin-Ultrogel as described (24). All dtXPB protein, as well as XPB and other known TFIIH subunits, were present in the 0.4 M KCl fraction, designated Hep0.4. Portions of this fraction were directly incubated without further fractionation with anti-HA resin to purify dtXPB and associated proteins. After incubation, the anti-HA resin was extensively washed, and bound material was eluted by competition with excess HA peptide. Subsequently, the composition of the eluate was analyzed by SDS-polyacrylamide gel electrophoresis and staining with silver nitrate. In addition to dtXPB, we identified only eight polypeptides ranging in molecular mass from 80 to 34 kDa that specifically and consistently immunoprecipitated with dtXPB and are thus XPB-associated factors (Fig. 3*B*). These associated proteins were all identified as known TFIIH subunits by two criteria: (i) reactivity with monoclonal antibodies specifically recognizing TFIIH subunits; and (ii) exact co-migration with known TFIIH subunits in SDS-polyacrylamide gel electrophoresis (data not shown). Finally, the staining intensity of the dtXPB subunit, compared with the other subunits, suggests that the dtXPB protein was predominantly present in stoichiometric amounts and not in a free form.

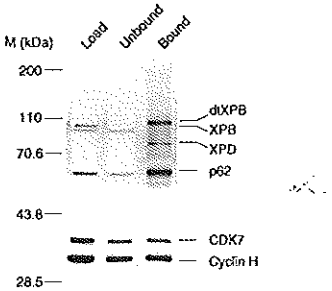


FIG. 2. **Immunoblot analysis of TFIIH factors associated with dtXPB in XP-t3 WCE using HA-antibody immunoprecipitation; dtXPB is incorporated in the TFIIH complex.** Indicated are the *load* (WCE), *unbound* (supernatant), and *bound* material (eluted with excess HA peptide). Because the amount of TFIIH was smaller in the lanes containing the load and unbound material, XPD appears as a very weak band in these two lanes as compared with the lane containing the bound fraction. The positions of the molecular weight markers used and the proteins detected by immunoblotting are indicated.

Presence of Additional NER and Transcription Factors in the Affinity-purified TFIIH Fraction—A number of NER and basal transcription factors (TFIIE, CSA, CSB, and XPG, among others) have been described to interact with the TFIIH complex, either as part of a RNA polymerase II holoenzyme (31, 32) or using isolated proteins (33, 34). Therefore, it was unexpected to find only nine polypeptides stained by silver nitrate in the immunopurified fraction. The silver-stained protein profile of affinity-purified TFIIH (Fig. 3*B*) does not exclude the possibility that substoichiometric amounts of other NER or transcription factors are present. Furthermore, the heparin fractionation might have disrupted salt-sensitive interactions. Therefore, the TFIIH-containing fraction that was immunoprecipitated directly from a WCE using low salt conditions was tested by immunoblot analysis for the presence of additional NER and transcription factors (Fig. 4). However, we were not able to detect the presence of any of the NER factors ERCC1, XPC, HHR23B, XPG, and CSB, RNA polymerase II, or any significant levels of the basal transcription factors tested. The absence of a stable interaction with CSB was confirmed by the reverse experiment, in which tagged CSB was immunoprecipitated and analyzed for the presence of TFIIH subunits (46). As a positive control, we detected the presence of the human homologue of yeast SUG1, a protein that we recently identified to interact with the XPB subunit of TFIIH (35). Notably, human SUG1 was below immunodetection level in the immunopurified fraction shown in Fig. 3*B*, indicating that this interaction was salt-sensitive under the conditions used. Similar results were obtained using fractionation of the WCE on a Ni-NTA column that has a high affinity for the hexahistidine stretch or by using a nuclear extract preparation (data not shown). The above results indicate that the interactions of TFIIH with the various NER and transcription factors are, at least under the various conditions we used, not stable.

Functional Characterization of Affinity-purified TFIIH—To determine whether the nine polypeptides identified in the silver-stained gel represent the active form of TFIIH, we tested whether the enzymatic DNA-dependent ATPase, DNA helicase, and CTD kinase activities that are associated with TFIIH were present in our purified preparations (27, 36). As shown in

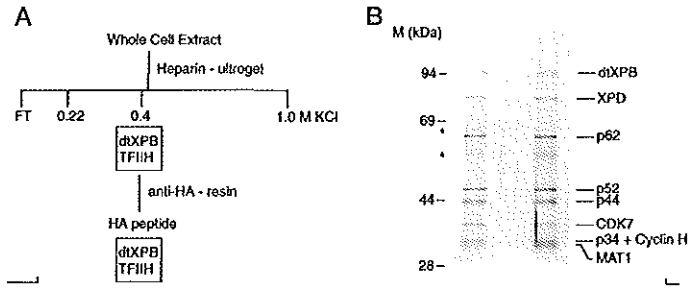


FIG. 3. **Immunopurification of dtXPB protein and associated factors.** *A*, schematic representation of the purification strategy. *B*, analysis of two independent anti-HA eluate preparations (10 μl) by 11% SDS-polyacrylamide gel electrophoresis stained with silver nitrate. The bands marked with asterisks (*) are also present in the empty lane and represent staining artifacts.

Fig. 5*A*, the ATPase activity detected was dependent on the presence of DNA and strongly stimulated by either circular M13 single-stranded or double-stranded supercoiled plasmid DNA. In addition, the DNA helicase and CTD kinase activities were readily detected (Fig. 5, *B* and *C*). In contrast, we were not able to detect any DNA nicking or exonuclease activity in the anti-HA fraction (data not shown).

Next, we tested the transcriptional activity of the anti-HA eluate (Fig. 5*D*). Addition of the purified complex was absolutely required in a fully defined reconstituted RNA polymerase II transcriptional reaction using the adenovirus 2 major late promoter. The anti-HA eluate contained only detectable TFIIH activity because omitting either TBP, TFIIB, TFIIE, or TFIIF abolished transcription completely, whereas omitting TFIIA resulted in a strongly decreased signal, in agreement with a stimulatory role for TFIIA in defined transcription reactions (Fig. 5*D*). These findings indicate the absence of detectable contaminating transcriptional activities in the TFIIH preparation. A quantitation of the enzymatic and transcriptional assays is presented in Fig. 5, *E* and *F*.

Finally, we characterized the activity of the affinity-purified TFIIH in *in vitro* NER assays. As shown in Fig. 6*A*, the affinity-purified complex was able to complement the NER defect in XP-B extracts. Quantitation of the incorporated labeled nucleotides in the damaged DNA allowed us to estimate the yield of active TFIIH, which was calculated to be ~12% (Table I), indicating that the anti-HA elution is rather efficient. Theoretically, the *in vitro* complementation could also be due to exchange of XPB with other TFIIH subunits. Therefore, we performed an antibody depletion experiment. Using anti-p62 antibodies, a HeLa WCE was depleted of TFIIH, which resulted in a co-depletion of NER activity (Fig. 6*B*, compare *lane 1* with *lanes 6* and *7*). As shown (Fig. 6*B*, *lanes 2–5*), the anti-HA eluate restored NER activity in the depleted HeLa WCE up to the level of the nondepleted extract, indicating that TFIIH complex formation involving XPB and p62 is essential for NER activity (25).

To analyze in more detail the NER activities of the purified TFIIH *in vivo*, a microneedle injection experiment was carried out. As anticipated, a strong stimulation of unscheduled DNA synthesis was readily observed directly after microinjection in XP-B and XP-D polykaryons, which was specific because it was not seen in XP-F and XP-G cells (Fig. 7, *A* and *C*). Interestingly, correction was also observed in TTD-A cells (Fig. 7, *B* and *C*) that contain a mutation in an as yet unidentified NER factor (37), in agreement with our earlier findings using highly purified TFIIH from HeLa cells by classical purification (8). These experiments indicate that TTDA either is an intrinsic component of TFIIH or is required for a modification of TFIIH, enabling it to function in repair (see "Discussion").

In conclusion, these experiments show that the immunopurification of dtXPB and associated proteins result in the rapid and efficient purification of TFIIH, which is active in NER and transcription.

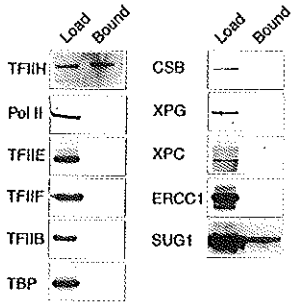
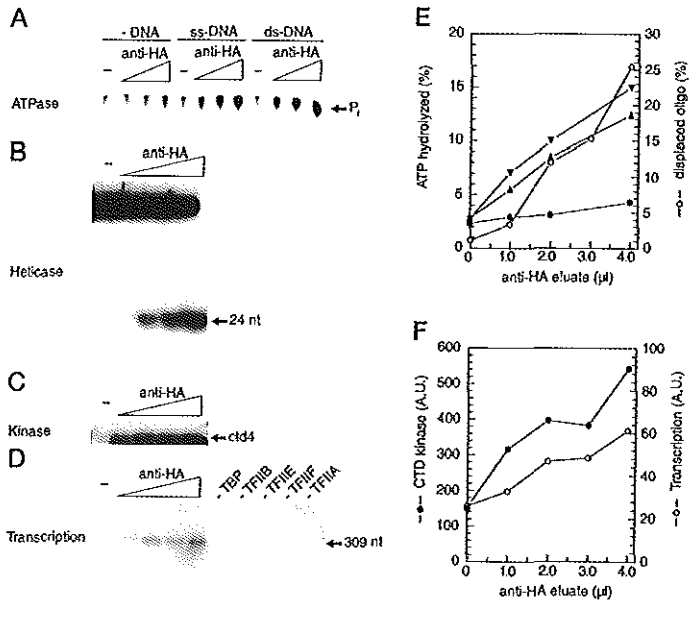


FIG. 4. **Association of TFIIH with NER and transcription factors.** None of the tested NER and/or basal transcription factors showed detectable association with TFIIH immunoprecipitated using HA-antibodies. As a positive control, human SUG1 was identified in the bound fraction. Antibodies used to identify multisubunit complexes recognized the p62 subunit of TFIIH, the RPB1 subunit of RNA polymerase II, the 34-kDa β-subunit of TFIIE, and the RAP74 subunit of TFIIF. Indicated are the *load* (WCE) and the *bound* fraction (eluted with excess HA peptide).

FIG. 5. **Analysis of TFIIH enzymatic and transcriptional activities in anti-HA eluates.** *A*, ATPase activity is strongly stimulated by single-stranded DNA (*ss-DNA*, M13mp18, 150 ng) or double-stranded DNA (*ds-DNA*, plasmid DNA, 150 ng). Increasing amounts of anti-HA affinity-purified TFIIH were used to measure ATPase activity (1, 2, and 4 μl). *B*, DNA helicase activity detected by displacement of a 24-mer oligonucleotide from M13mp18 single-stranded DNA. Indicated are increasing amounts of anti-HA eluate (1, 2, 3, and 4 μl). *C*, CTD kinase activity by increasing quantities of anti-HA eluate (1, 2, 3, and 4 μl). *D*, TFIIH transcriptional activity is present in anti-HA eluate. Indicated are increasing amounts of anti-HA eluate (0, 1, 2, 3, and 4 μl) added to a complete reaction or individual transcription factors omitted from the reaction containing anti-HA eluate (4 μl). *E*, quantitation of the ATPase and DNA helicase activities. The ATPase activity is represented by the percentage of ATP hydrolyzed in the absence of DNA (●), and in the presence of single-stranded DNA (▼) and double-stranded DNA (▲). The helicase activity is depicted by the percentage oligonucleotide displaced from the single-stranded DNA (○). *F*, quantitation of the CTD kinase (●) and transcriptional activities (○). Both the CTD kinase and transcription are represented in arbitrary units (*A.U.*).



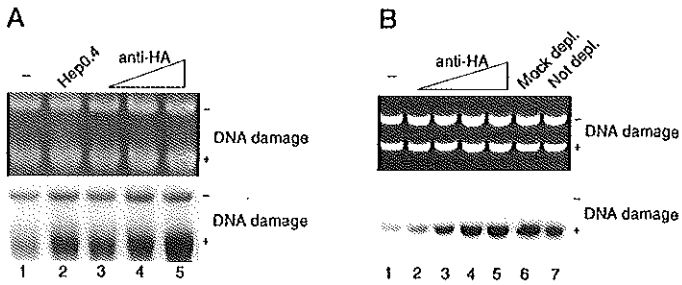


FIG. 6. **NER activity of affinity-purified TFIIH *in vitro*.** *A, in vitro* complementation of XP-B NER-deficient WCE (150 μg) by fractions from the XPB purification (*lane 1*, XP-B extract alone; *lane 2*, XP-B extract with 2 μl of Hep0.4 fraction; *lanes 3–5*, XP-B extract containing 2, 5, and 10 μl, respectively, of anti-HA fraction). *Upper panel*, ethidium bromide-stained gel; *lower panel*, autoradiogram. *B*, depletion of NER activity from HeLa WCE (100 μg/reaction) with anti-p62 monoclonal antibodies and restoration of NER activity with anti-HA eluate. *Lane 1*, depleted extract; *lanes 2–5*, depleted extracts containing 2, 4, 6, and 8 μl of anti-HA eluate, respectively; *lane 6*, mock-depleted extract; *lane 7*, nondepleted extract. *Upper panel*, ethidium bromide-stained gel; *lower panel*, autoradiogram.

TABLE I
Purification of the TFIIH complex using the dtXPB protein

Fraction	Protein	Activity[a]	Yield
	mg	*units*	%
WCE	54.4	—[b]	
Hep0.4	17.5	1731	100
Anti-HA	—[c]	208	12

[a] One unit of activity was defined as the amount of protein used to increase the incorporation 2-fold relative to the receiving XP-B extract (45).

[b] Activity of the WCE could not be determined.

[c] Protein concentrations were determined using the BCA protein assay (Pierce) and could not be reliably determined for the anti-HA fraction.

DISCUSSION

TFIIH was originally purified as a basal transcription factor from rat, yeast, and human (24, 38–40) and was first shown by Schaeffer *et al.* (9) to be involved in NER; this involvement was subsequently demonstrated by others as well (25, 33, 41). By immunopurifying the XPB protein using a cell line expressing functional tagged XPB, we describe an improved and facilitated purification for TFIIH free of contaminating NER and transcriptional activities that is an efficient, essentially one-step, procedure utilizing physiological elution conditions.

Utilizing this protocol, which avoids the high salt and hydrophobic chromatography conditions of the classical purification procedure, we identify TFIIH as a nine-subunit complex. In addition, we show that each complex contains only one molecule of the XPB helicase. The intensity of protein staining of the XPD subunit in the purified complex compared with XPB is consistent with the idea that the XPD helicase, also, is present on a molar basis in the complex. The occurrence of two helicase molecules per TFIIH complex is in agreement with the concept that the functional forms of a number of helicases are oligomers, generally dimeric or hexameric (42). As reported previously, CDK7 and cyclin H, together with MAT1, are part of both TFIIH and a separate trimeric complex in the cell (13). This is consistent with our findings in the immunoprecipitation experiment that CDK7 and cyclin H are relatively more abundant in the WCE and unbound fraction as compared with the XPB-associated fraction (see Fig. 2).

During the past years, all eight XPB-associated factors were identified and cloned as TFIIH subunits purified from both HeLa cells and from the budding yeast *Saccharomyces cerevisiae* (16, 43), suggesting that in solution in repair- and transcription-competent WCEs, at least the major form of TFIIH is composed of nine subunits and is active in NER, as well as transcription. However, the possibility cannot be excluded that substoichiometric and/or poorly stained subunits are essential for, for example, NER functioning, and therefore, definite proof that both the NER and transcriptional activity of TFIIH resides with the nine identified and cloned subunits awaits re-

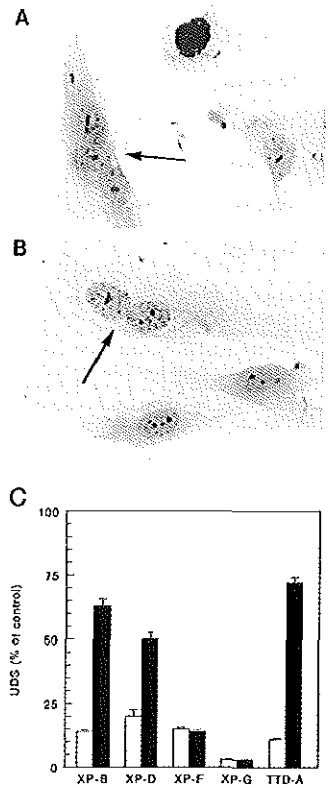


FIG. 7. **Correction of NER defect by microinjected affinity-purified TFIIH in XP-B, XP-D, and TTD-A cells *in vivo*.** *A* and *B*, micrographs showing the effect on NER activity of injection of the anti-HA eluate in XP-B (*A*) and TTD-A (*B*) cells. The injected fibroblasts (polynuclear, obtained by cell fusion prior to injection) are indicated by *arrows*. The heavily labeled cell in *A* is a noninjected S phase cell. *C*, quantitation of NER activity of injected XP and TTD-A polykaryons. The *bars* represent the average unscheduled DNA synthesis (*UDS*) level (obtained by counting grains above 50 nuclei), and S.E. values are indicated. As a control, unscheduled DNA synthesis of parallel-treated uninjected wild type fibroblasts (C5RO) were counted and arbitrarily set at 100%. *Open bars*, noninjected cells; *closed bars*, injected cells.

constitution of TFIIH from recombinant proteins.

One of the TFIIH factors that is not yet assigned to a subunit is TTDA. We are presently investigating whether any of the known TFIIH genes are mutated in TTD-A cells. However, it is theoretically also possible that TTDA is not a subunit of the TFIIH complex itself but is implicated in TFIIH modification as part of its function. Recently, we have identified human SUG1 as a protein interacting with the XPB subunit of TFIIH (35). Little is known about posttranslational regulation of TFIIH function and the role of factors like SUG1 that are thought to unfold or refold proteins in the context of several processes, including regulated proteolysis. Like SUG1, TTDA could play a role in TFIIH modification without being part of the complex. The inability to generate high levels of dtXPB protein, even when the cDNA was expressed under control of strong promoters, suggests an autoregulatory mechanism of XPB protein levels. For example, a similar observation was made in the case of overexpression of the NER protein ERCC1, which forms a complex with XPF, and TBP, which is part of the basal transcription factor TFIID (22, 44).

Using NER- and transcription-competent WCEs, physiological washing conditions, and nonoverexpressed functional dtXPB protein, we failed to observe, within our limits of detection, interactions with any NER and/or transcription factor tested, although some of them were reported previously by others (31–34). Several explanations can be put forward for this apparent discrepancy. Many methods for identification of protein-protein interactions use overexpressed, *in vitro* synthesized or purified proteins often involving heterologous expression systems. When the protein normally resides in a complex and has multiple interaction domains, it may exhibit promiscuous association behavior when studied in isolation because of the lack of its natural partners, improper folding, or lack of posttranslational modification. Alternatively or in addition, the interactions observed were not stable during our extract preparations and/or were transient or induced upon DNA binding.

The procedure described here greatly facilitates the isolation of active TFIIH. It is simple, fast, and reproducible and does not require extensive chromatographic procedures. In combination with specific procedures for extract preparation, it may be exploited further for the purification of holo-complexes involved in NER and/or transcription. Furthermore, this procedure may allow the isolation of TFIIH with mutated XPB subunits for biochemical analyses to obtain more insight into the requirements for the XPB helicase-mediated function in NER and transcription initiation. Because the reconstitution of TFIIH from recombinant source is lacking at this moment, it would also be of interest to add (epitope) tags to other TFIIH subunits for functional analysis of TFIIH with mutations in, for example, the second helicase subunit, XPD. These experiments are in progress.

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CHAPTER 3

TFIIH with Inactive XPD Helicase Functions in Transcription Initiation But is Defective in DNA Repair

Submitted for publication

TFIIH WITH INACTIVE XPD HELICASE FUNCTIONS IN TRANSCRIPTION INITIATION BUT IS DEFECTIVE IN DNA REPAIR

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TFIIH is a multisubunit protein complex involved in RNA polymerase II transcription and nucleotide excision repair, a DNA repair mechanism important for removal of a wide variety of DNA lesions including UV-induced photoproducts. Mutations in the largest subunits of TFIIH, the DNA-dependent ATPases and helicases XPB and XPD, are associated with three inherited syndromes: xeroderma pigmentosum with or without Cockayne syndrome, and trichothiodystrophy. Using epitope-tagged XPD we purified mammalian TFIIH carrying a wild type or an XPD active-site mutant subunit. In contrast to XPB, XPD helicase activity was dispensable for *in vitro* transcription, catalytic formation of tri-nucleotide transcripts and promoter opening. Moreover, microinjection of mutant XPD cDNA did not interfere with *in vivo* transcription. These data show directly that XPD activity is not required for transcription. However, with respect to DNA repair, neither 5' nor 3' incisions in defined positions around a DNA adduct were detected in the presence of TFIIH containing inactive XPD, despite the fact that substantial damage-dependent DNA synthesis is induced in the presence of mutant XPD both *in vitro* and *in vivo*. This indicates that mutant XPD causes aberrant damage-dependent DNA synthesis without effective repair, consistent with the discrepancy between repair synthesis and survival in a number of cells from XP-D patients.

INTRODUCTION

Nucleotide excision repair (NER) is one of the most versatile DNA-damage repair mechanisms in eukaryotic cells. A wide variety of DNA lesions, including UV-induced photoproducts and bulky chemical adducts can be removed by this multi-enzyme system. After recognition of a damaged site, a region around the lesion is locally unwound, which is followed by the formation of two incisions around a lesion. The subsequent removal of the damaged oligonucleotide is succeeded by gap-filling DNA synthesis and ligation to restore the original DNA sequence (1-3). Six protein factors are required for the early incision stage (4,5). The XPC-hHR23B complex, which binds with high affinity to DNA damage and represents the primary damage recognition factor (6), XPA, RPA, and transcription factor IIH (TFIIH) are involved in verification of the lesion, ATP-dependent local unwinding of DNA around the damage and organization of the remainder of the NER complex (7,8). This provides a substrate for the structure-specific endonucleases ERCC1-XPF and

XPG, which incise the damaged strand asymmetrically on the 5' and 3' sides, respectively.

In addition to its role in NER, TFIIH is one of five basal transcription factors required for accurate initiation of transcription of protein coding genes by RNA polymerase II (9,10) (see for review (11)). In the initiation reaction, TFIIH is required for the ATP-dependent formation of melted regions around the transcription start site (12). This requirement is alleviated by a pre-melted promoter region (12,13) or negative supercoiling which can induce transient DNA melting (14,15). TFIIH consists of nine subunits for which all genes have been cloned (16,17). Several enzymatic activities have been identified for TFIIH and attributed to individual subunits: both of the largest two components, XPB and XPD, are DNA-dependent ATPases and DNA helicases (18-20), while CDK7 is a protein kinase which is capable of phosphorylating the C-terminal domain of the largest subunit of RNA polymerase II (21). Non-lethal defects in the two helicase subunits, XPB and XPD, give rise to the inherited disorders xeroderma pigmentosum (XP), Cockayne's syndrome (CS) combined with XP, or trichothiodystrophy (TTD) (22). Some of the clinical features of these syndromes, such as UV sensitivity and predisposition to skin cancer (characteristic for XP), may be due to defective functioning of TFIIH in NER, while other symptoms, such as severe growth retardation, neurodysmyelination (XP/CS and TTD), and brittle hair (TTD), may be caused by a subtle defect in the transcriptional activity of TFIIH (see (23) and (24) for further explanation). At the cellular level, mutations in the XPD gene frequently result in relatively high levels of damage-dependent DNA synthesis which does not generally correlate with residual repair activity and UV sensitivity (25).

To obtain insight into the biochemical defects associated with mutations in TFIIH, we examined the contribution of the XPD-associated enzymatic activities to TFIIH function. An XPD mutant was used with a defined single amino acid change in the Walker type A nucleotide-binding motif (consensus GXGKT), lysine to arginine at position 48 (K48R), which preserves the positive charge. Biochemical analysis of the corresponding mutation in the *S. cerevisiae* homolog of XPD, Rad3p, has demonstrated that this mutation dramatically impairs ATP hydrolysis, although the protein retains the ability to bind ATP (26). Yeast cells carrying the same mutation are viable, but UV-sensitive (26,27).

In this report we describe the effect of an active-site XPD mutant on mammalian TFIIH function. We provide direct evidence that XPD helicase activity is dispensable for basal transcription. In contrast, we show that XPD helicase activity is required for the formation of both the 5' and 3' incision around a site of DNA damage.

MATERIALS AND METHODS

Oligonucleotides, site-directed mutagenesis and plasmid DNA construction

The mutation in the nucleotide binding domain encoded by the XPD cDNA was generated by site-directed mutagenesis using oligonucleotide p142 5'-TCAGG-CACCGGGCGAACAGTATCCCTG-3'. The desired mutation was verified by sequence analysis and XPD wild type and mutant cDNAs were cloned as *Eco*RI fragments into eukaryotic expression vector pSVL, a modified pSVM vector (Pharmacia), yielding plasmids pM52 and pM124; and pcDNA3 (Invitrogen), yielding plasmids pM264 and pM265. The C-terminal HA-epitope tag was added by PCR using primers p117 5'-GCCGAAACCAGTGTCGCCCT-3' (5' primer) and p240 5'-AGGAATTCTCAGCTAGCGTAATCTGGAACATCGTATGGGTAT-CCGAGCTGCTGAGCAATCTGCTC-3' (3' primer). PCR products were cloned into *Sac*II-*Eco*RI sites of pBluescript (Stratagene) and sequenced to insure the absence of any PCR-generated errors. Eukaryotic expression vectors containing cDNA encoding wild type and K48R mutant HA-tagged XPD were generated by ligation of the *Eco*RI-*Sca*I and the *Sca*I-*Eco*RI cDNA fragments into the *Eco*RI site of pcDNA3, yielding plasmids pM435 and pM436.

Isolation of TFIIH containing wild type and K48R mutant XPD

Cell lines UV435.12 and UV436.1 expressing wild type and K48R mutant HA-tagged XPD cDNA, respectively, were generated by transfection of CHO UV5 cells with plasmids pM435 and pM436 using Lipofectin reagent (Life Technologies) essentially as suggested by the manufacturer. After selection with G418 (0.75 mg/ml), independent clones were isolated and tested for expression by immunoblotting of cell lysates using anti-HA 12CA5 monoclonal antibodies. Whole cell extracts (WCEs) were prepared from 3 L of suspension culture of either cell line (28) and loaded onto a heparin-Ultrogel column (IBF, France) equilibrated in buffer A (25 mM Hepes-KOH (pH 7.8), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol) containing 0.1 M KCl and step eluted in buffer A containing 0.1 M, 0.22 M, 0.4 M, and 1.0 M KCl. The 0.4 M KCl fraction containing all of the TFIIH was incubated for 16 h with 100 µl protein G-sepharose beads (Pharmacia) and 250 µg monoclonal antibody 12CA5, essentially as described before (29). The resin was recovered by centrifugation (3 min, 1000 rpm), and washed 3 times in buffer T (50 mM Tris-HCl (pH 7.9), 0.2 mM EDTA, 0.5 mM DTT, 17% (v/v) glycerol) containing 0.4 M KCl and 0.1% Nonidet P-40 and twice in buffer T containing 0.1 M KCl and 0.01% Nonidet P-40. Bound TFIIH was eluted from the resin in two washes with the latter buffer supplemented with 2.0 mg/ml HA-peptide (YPYDVPDYA) for 1 h at 30 °C. TFIIH was dialysed to remove the peptide in buffer T containing 0.05 M KCl and 0.01% Nonidet P-40 for enzymatic and NER

analysis or buffer A (20 mM HEPES-KOH pH 7.9, 17% glycerol (v/v), 1 mM EDTA, 1 mM DTT, and 0.1 M KCl; BSA was added to a final concentration of 0.2 mg/ml) for transcriptional analysis and stored in aliquots at -80°C. During purification, all procedures were carried out at 4 °C, except where noted otherwise.

Analysis of TFIIH-associated enzymatic activities

Purified TFIIH was analyzed for ATPase, CTD kinase and DNA helicase activities as described before (29).

Analysis of in vitro transcription activity

Analysis of transcription activity was carried out with highly purified or homogeneous preparations of recombinant human TBP, TFIIB, TFIIE, TFIIF, and affinity-purified RNA polymerase II from calf thymus as detailed before (12,15). Reactions (20 µl volume) contained 12 ng TBP, 50 ng TFIIB, 80 ng TFIIF, 30 ng RNA polymerase II, 75 ng TFIIE, and TFIIH as indicated, and contained 12 mM HEPES-KOH (pH 7.9), 60 mM KCl, 5 mM $MgCl_2$, 3 mM $(NH_4)_2SO_4$, 0.6 mM EDTA, 0.7 mM DTT and 120 µg/ml BSA.

Standard transcription reactions contained 100 ng linearized pΔML(200) template (14) containing the adenovirus major late promoter, 60 µM ATP, 60 µM UTP, 10 µM [α-^{32}P]CTP (80 Ci/mmol), and 120 µM 3'-OMeGTP, as described (15).

For analysis of tri- and 15-nucleotide RNA product formation, 5 ng of respectively AdML+3G or AdML+15G template DNA (372 bp *Pvu*II fragment) were used (30). Partial non-template DNA sequences are (underlined are the natural transcription start site and the first G residue):

AdML + 3G: 5' - CCTCACGCTCTTCCTCTAGAGTC - 3'

AdML + 15G: 5' - CCTCACTCTCTTCCTCTAGAGTC - 3'

After pre-incubation in the above described reaction mixture for 45 min at 30 °C, nucleotides were added (60 µM ATP, 3 µM [α-^{32}P]CTP (267 Ci/mmol), 10 µM UTP, 120 µM 3'-OMeGTP, and reactions were incubated for the indicated time. Reactions were analyzed as described by 20% polyacrylamide (acrylamide:bisacrylamide=19:1)-8.3 M urea gelelectrophoresis, after inactivation of RNA polymerase II by heating for 3 min at 68 °C and alkaline phosphatase treatment (30).

$KMnO_4$ sensitivity assay of transcription intermediates

For the $KMnO_4$ analysis of open complex formation, radiolabeled probe (0.2-0.4 ng *Eco*RI-*Hind*III AdML+15G fragment, labeled at the non-template strand (12)) was incubated in the above reaction mixture for 45 min at 30 °C. After this pre-

incubation, nucleotides (60 μM ATP, 10 μM CTP, 10 μM UTP, and 120 μM 3'-OMeGTP) were added and incubated for 10 min at 30 °C. Two μl of a freshly prepared 160 mM $KMnO_4$ was added for 20 seconds after which reactions were stopped by addition of 2 μl of 14.4 M β-mercaptoethanol. DNA was cleaved at modified residues by piperidine and analyzed on 7% polyacrylamide (acrylamide:bisacrylamide=19:1)-8.3 M urea gel electrophoresis as described (12,30).

NER in vitro - DNA synthesis assay

WCE were prepared from repair-proficient cell lines CHO AA8, and repair-deficient CHO NER mutant UV5. Reactions contained plasmid randomly damaged with *N*-Acetyl-AAF and an equal amount of undamaged control plasmid with a different size, the indicated amounts of extracts and purified protein, and labeled nucleotides as described before (31,32). After incubation for 3 h at 30 °C, DNA was isolated, linearized and analyzed by agarose gel electrophoresis.

Nucleotide excision repair in vitro - detection of incision products

Covalently closed circular DNA containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink (Pt-GTG) was produced as described (5). Repair was carried out in 8.5 μl reaction mixtures for the fully defined reconstituted reactions or in 10 μl reaction mixtures for the cell extract complementation. Repair reactions were carried out in buffer containing 45 mM HEPES-KOH (pH 7.8), 70 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.3 mM EDTA, 10% glycerol, 2.5 μg BSA and 2 mM ATP. Complementation reactions were supplemented with 40 mM phosphocreatine (di-Tris salt) and 0.5 μg creatine kinase. Each reconstituted reaction contained 50 ng RPA, 45 ng XPA, 10 ng XPC-hHR23B complex, 50 ng XPG, 20 ng ERCC1-XPF complex and either 1.5 μl of HeLa TFIIH Hep fr. IV (33) or 1.5 μl and 3 μl TFIIH containing wild type or K48R mutant XPD subunit. Following pre-incubation for 10 min at 30 °C, 50 ng Pt-GTG were 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. Six ng of an oligonucleotide was added, complementary to the excised DNA fragment and with 4 extra G residues at the 5' end. The excision products were radiolabeled with 0.1 units of Sequenase v2.0 polymerase and 1 μCi[α-^{32}P]dCTP, separated on a 14% denaturing polyacrylamide gel and visualised by autoradiograph as described (34).

For the detection of 3' and 5' incisions, the (Pt-GTG) plasmid was digested with *Ava*II at 140 bp 3' from the lesion, radiolabelled using DNA polymerase I (Klenow fragment) and [α-^{32}P]dTTP, and purified over a G-50 Sephadex column. Repair reactions were performed as in the dual incision assay and DNA products

were analyzed by denaturing 6% polyacrylamide gel electrophoresis as described previously (7).

Microneedle injection of cDNA expression vectors and assay for RNA synthesis and UV-induced unscheduled DNA synthesis (UDS)

Microinjection of cDNA expression plasmids in nuclei of XP-D primary fibroblasts (XP1BR and XP6BE) as well as control cells (C5RO) was performed as described previously (35). Briefly, at least three days prior to injection cells were fused with inactivated Sendai virus, and seeded onto coverslips. After nuclear injection of cDNA constructs, cells were incubated in normal culture medium for 24 h to allow expression of the injected DNA molecules. Subsequently, UDS was determined after UV-C irradiation (15 J/m^2), incubation for 2 h in [^{3}H]thymidine-containing culture medium, fixation and *in situ* autoradiography. Grains above nuclei were counted. RNA synthesis was determined by counting autoradiographic grains above the nuclei of injected cells, after labeling with [^{3}H]uridine during a pulse labeling period of 1 h in normal culture medium.

UV-survival and analysis of unscheduled DNA synthesis of transfected cells

Transfections of CHO UV5 (36) and human HD2 (37) cells with plasmids pM265 and pM266 were performed using lipofectin reagent (Life Technologies) essentially as described by the manufacturer. After selection with G418 (0.75 mg/ml for CHO cells; 0.3 mg/ml for HD2 cells), single clones were isolated and selected for expression of wild type and mutant XPD by immunoblot analysis. UV survival was assayed using [^{3}H]thymidine incorporation (38). Briefly, 3 days after irradiation with UV, proliferating cells were pulse-labeled with [^{3}H]thymidine (4 h), followed by a chase (2 h). Cells were washed and lyzed in 0.05 N NaOH. Quantification of the [^{3}H]thymidine incorporation by liquid scintillation counting provides a measure of cell viability. For analysis of unscheduled DNA synthesis, cells were plated onto glass coverslips. After 24 h, cells were irradiated, pulse-labeled in [^{3}H]thymidine-containing culture medium for 2 h, fixed and subjected to *in situ* autoradiography (35).

RESULTS

Isolation of TFIIH complexes containing wild type and K48R mutant XPD subunit

To investigate the role of ATP hydrolysis by the XPD helicase subunit of TFIIH during NER and transcription initiation, we isolated TFIIH complexes containing wild type and mutant XPD protein. A cDNA encoding wild type XPD containing a C-terminal HA-epitope tag and a cDNA encoding a similarly HA-tagged K48R mutant protein were generated. In the mutant, the highly conserved lysine residue in the Walker type A nucleotide binding motif was replaced by the similarly charged arginine (GKT→GRT, see Fig. 1*A*). UV-sensitive, NER-deficient Chinese hamster ovary (CHO) cells from complementation group 2 (UV5), carrying a mutation in the XPD gene, were transfected with cDNA encoding wild type and K48R protein, respectively, and independent clones stably expressing HA-tagged XPD were isolated. Using anti-HA monoclonal antibodies, immunoblot analysis of cell lysates from two selected clones expressing wild type and mutant HA-tagged XPD, respectively, shows a specific band at the expected molecular mass of XPD (Fig. 1*B*). The protein level of the wild type and K48R mutant appeared comparable.

The ability of HA-tagged XPD to correct the defective repair of UV5 cells was assessed by determining the UV survival of the transfected cells. The results presented in Fig. 1*C* show that a cDNA encoding HA-tagged XPD is able to confer UV-resistance to UV5 cells close to the level of untagged XPD cDNA and in the range of the parental repair-proficient cell line AA8. This indicates that addition of the HA-epitope tag does not negatively influence the function of the XPD protein in NER.

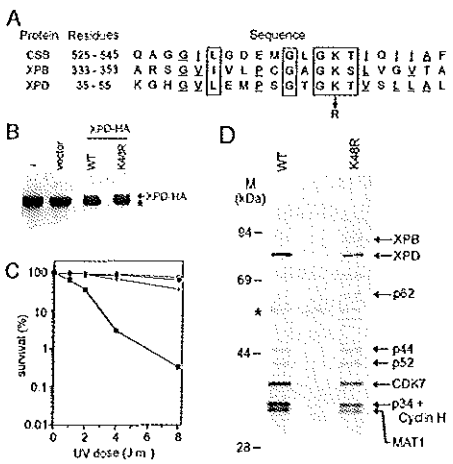

Figure 1. Purification of wild type and K48R XPD-containing TFIIH complex from mammalian cells. *A*, Homology of the conserved Walker type A nucleotide-binding motif of XPD with the sequences of the XPB and CSB proteins. Identical or similar residues are underlined; residues of the consensus nucleotide-binding motif are boxed. Indicated is the introduced point mutation at position 48. *B*, Immunoblot analysis of CHO UV5 transfectants using anti-HA 12CA5 monoclonal antibodies. Lysates were obtained from untransfected (-), G418-resistant cells transfected with empty vector (vector), and cells transfected with human cDNA encoding XPD-HA wild type (WT) and XPD-HA K48R protein. The position of HA-tagged XPD is indicated with an arrow, cross-reactive material that cannot be precipitated by 12CA5 antibodies is marked with an asterisk. *C*, UV-survival analysis of CHO UV5 mutant cells (■), transfected with cDNA encoding (untagged) human XPD (•), and HA-tagged XPD (+)

protein, and the parental wild type cell line AA8 (o). *D*, Purified wild type (WT) and K48R XPD-TFIIH complexes were subjected to 11% SDS-PAGE and proteins were visualised by staining with silver nitrate. Indicated are the positions of the molecular mass markers, and the TFIIH subunits identified on the basis of apparent molecular mass. The identities of p44 and Cyclin H were confirmed by immunoblotting using antibodies that cross-react with Chinese hamster TFIIH factors (not shown). The band corresponding to the p52 subunit could not be assigned conclusively because antibodies raised against the human protein did not cross-react. Keratin artifact bands are marked with an asterisk.

To obtain highly purified wild type and K48R XPD complexes from whole-cell extracts (WCE), a fast and efficient two-step purification protocol was employed previously developed for the purification of HA-tagged XPB containing TFIIH. This protocol consists of chromatography on heparin-Ultrogel and anti-HA immunosorption followed by peptide elution (29). Isolated complexes were subjected to SDS-PAGE and stained with silver nitrate (Fig. 1*D*). In addition to XPD, proteins with molecular masses ranging from 32-90 kDa were identified corresponding to all nine known TFIIH subunits as is indicated in the figure (Fig. 1*D*). As expected, the bands corresponding to XPD and the three CAK components (CDK7, Cyclin H, and MAT-1) were more prominent as compared to the other TFIIH subunits. This is in agreement with the finding that XPD resides in two complexes: TFIIH and XPD-CAK (39). We were not able to efficiently separate XPD-CAK and TFIIH by subsequent chromatography as their purification properties are very similar. However, since the staining pattern of the wild type and K48R mutant fractions were almost identical, the activities of the HA fractions could be directly compared.

Comparison of TFIIH-associated enzymatic activities of wild type and K48R mutant XPD complexes

Several enzymatic activities are associated with TFIIH (40,41). As is shown in Fig. 2*A*, the ATPase activity of the K48R complex is reduced to about half of wild type level. Most likely, the residual activity measured is derived from the second DNA-dependent ATPase subunit of TFIIH, XPB. This result suggests that the XPD subunit contributes significantly to the ATPase activity of TFIIH.

Secondly, the DNA helicase activities were compared using a radiolabeled 24-mer oligonucleotide annealed to single-stranded M13 DNA. This substrate design allows measurement of DNA helicase activity independent of helicase polarity. As is shown in Fig. 2*B*, wild type XPD complex is able to release annealed oligonucleotide from the M13 DNA. However, the mutant K48R XPD complex added in the same quantity appears to be completely deficient in the ability to release the labeled oligonucleotide. This result indicates that the XPD DNA helicase is absolutely required for the DNA-unwinding activity of TFIIH measured in this assay, irrespective of DNA polarity. The fact that both the TFIIH-associated ATPase and DNA helicase activities are severely affected is consistent with the notion that the introduced point mutation in the nucleotide binding motif of XPD abolishes its associated enzymatic activities.

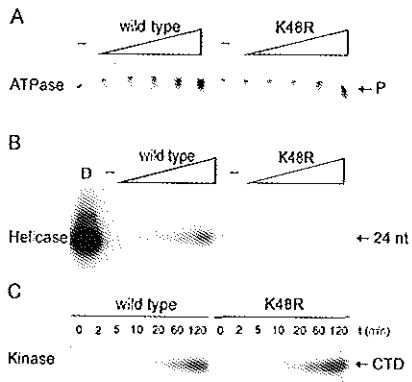

Figure 2. Comparison of TFIIH-associated enzymatic activities of wild type and K48R XPD complex. *A*, Autoradiogram showing reduction of DNA-dependent ATPase activity of K48R XPD complex. Radiolabeled ATP was incubated in the presence of 150 ng M13 single-stranded DNA with increasing amounts of XPD complex (0, 0.5, 1, 2, 3, and 4 μl) and the released phosphate was separated by thin-layer chromatography. *B*, DNA helicase activity is absent in K48R XPD complex. Helicase activity was measured in the presence of increasing quantities XPD complex (0, 0.5, 1, 2, 3, and 4 μl) as detected by the release of a radiolabeled 24-mer oligonucleotide from single-stranded M13 DNA. Δ indicates the heat-denatured control. *C*, CTD kinase activity is not affected by the K48R mutation in XPD. Activity was measured in the presence of wild type or K48R XPD complex (2 μl) using a peptide containing four repeats of the conserved repeat YSPTSPS of the large subunit of RNA polymerase II and incubated with radiolabeled ATP.

Finally, we characterized the kinase activity that is able to phosphorylate the carboxy-terminal domain of the largest subunit of RNA polymerase II, RPB1. Equal amounts of both wild type and K48R XPD complexes were incubated with a synthetic tetra-repeat heptapeptide corresponding to the conserved repeat of RPB1 in the presence of radiolabeled ATP. The CTD-peptide was phosphorylated in a time-dependent manner with similar efficiency by both wild type and K48R XPD complexes (Fig. 2*C*).

TFIIH containing inactive XPD ATPase subunit supports transcription initiation

To investigate whether the K48R effect on ATPase and helicase activity has consequences for the function of TFIIH in basal transcription, we tested both wild type and mutant XPD fractions in RNA polymerase II mediated *in vitro* transcription assays. Wild type and K48R XPD complexes were incubated in the presence of homogenous recombinant TBP, TFIIB, TFIIE, TFIIF and highly purified RNA polymerase II, linearized DNA template containing the adenovirus major late promoter, and ribonucleotides. Interestingly, correctly initiated transcripts were produced in the presence of either wild type and K48R XPD complex (Fig. 3*A*). This was specifically due to TFIIH activity in both wild type and K48R mutant preparations since omission of any basal factor from the reaction mixture did not yield appreciable transcript levels (Fig. 3*B*). The level of transcription induced by K48R mutant TFIIH varied between different preparations within a two-fold range as compared to wild type.

To further characterize the possible effect of the K48R mutation in the XPD subunit of TFIIH on transcription, we looked at early stages of transcription initiation. RNA products shorter than 11 nucleotides accumulate with time, due to

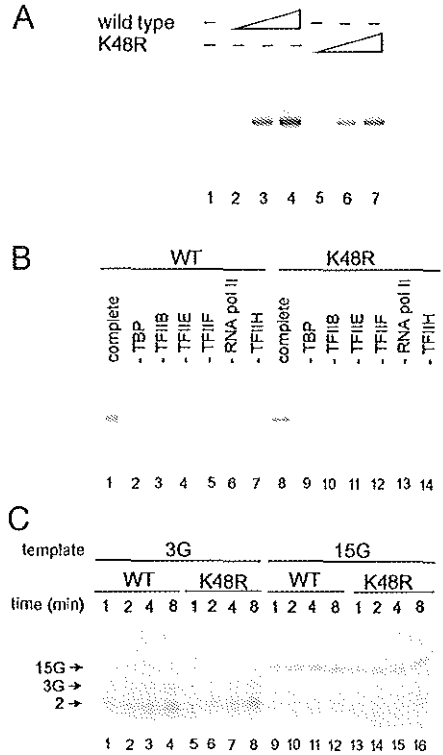

Figure 3. TFIIH containing inactive XPD DNA helicase allows transcription initiation by RNA polymerase II similar to wild type TFIIH. *A*, Reconstituted transcription reactions. Reactions contained purified recombinant basal transcription factors TBP, TFIIB, TFIIE, TFIIF, and purified RNA polymerase II (lanes 1-7), wild type (1, 3, and 5 µl, lanes 2-4, respectively) or K48R XPD complex (1, 3, and 5 µl, lanes 5-7, respectively) and linearized template DNA containing the AdML promoter and a G-less cassette of 200 bp. *B*, Stimulation of RNA polymerase II transcription by wild type and K48R complex is specifically due to TFIIH activity. Transcription reactions contained TFIIH (3 µl; wild type, lane 1-6, or K48R, lane 8-13) and all basal transcription factors and RNA polymerase II (lane 1 and 8), or a single factor omitted as indicated above the lanes (lanes 2-7, and 9-14). *C*, Time-course analysis of the formation of short RNA-products. Reactions contained basal transcription factors and RNA polymerase II (3 µl TFIIH; wild type, lane 1-4 and 9-12, or K48R, lane 5-8 and 13-16), and template DNA (AdML+3G, lane 1-8; AdML+15G, lane 9-16).

multiple rounds of abortive synthesis and initiation by the same RNA polymerase II complex (30). If subtle differences, such as the dynamic transition from closed to open conformation exist in the presence of mutant TFIIH, they may become apparent from analysis of small RNA products. Therefore, we incubated mutant and wild type TFIIH with template DNA in the presence of other basal transcription factors, RNA polymerase II and the appropriate nucleotides, and analyzed the formation of tri-nucleotides (with AdML+3G template, Fig. 3*C*, lane 1-8) and 15-nucleotide products (AdML+15G template, Fig. 3*C*, lane 9-16). As is evident from Fig. 3*C*, the products from the AdML+3G template, tri- and mainly di-nucleotides (30), increase with time due to multiple rounds of abortive synthesis and initiation. This is not observed with the AdML+15G template, which does not allow abortive initiation in agreement with previous results (30). Note that much more tri- and di-nucleotide products are formed, which incorporated only one (labeled) CTP, as compared to the 15-nucleotide product, which incorporated six CTPs. Importantly, however, no significant difference is observed in the formation of tri- and di-nucleotide, and 15-nucleotide transcripts in the presence of either wild type or mutant TFIIH.

Open complex formation in transcription is unaffected with inactive XPD helicase

Since TFIIH is implicated in melting of DNA during the transcription initiation event, we analyzed the promoter-template region for single stranded regions with permanganate. Basal transcription factors, wild type or K48R mutant TFIIH, and RNA polymerase II were incubated with template DNA to allow the formation of pre-initiation complexes. Subsequently, ATP was added to allow transient initial opening of the promoter region (12,30). The specific activity of the TFIIH preparation was not sufficient to allow detection of the unstable initial opening of the promoter region (Fig. 4, lane 1 and 2; 3 and 4). However, upon addition of ATP, UTP, CTP, and 3'-OMeGTP that allows the formation of a specific 15-nucleotide transcript, permanganate sensitivity of thymidine residues at position +5 to +13 was clearly enhanced (Fig. 4, compare lane 1 and 3 and 4 and 6). Importantly, the increased sensitivity of this region, which indicates the formation of an open region downstream the initiation site, was found in the presence of both wild type and mutant TFIIH containing an inactive XPD helicase subunit.

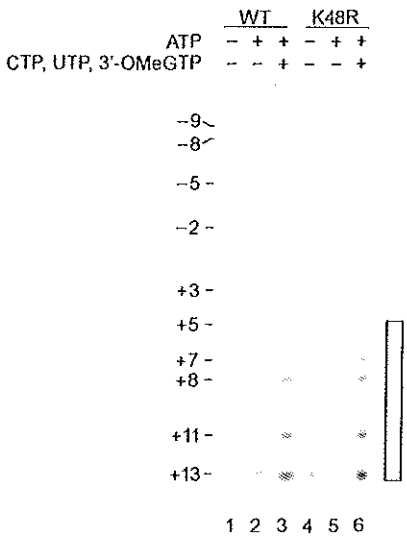

Figure 4. $KMnO_4$ sensitivity of the AdML promoter region around the start site. Open regions from +5 to +13 are efficiently detected in the presence of both wild type and mutant TFIIH (5 µl) after production of a 15-nucleotide transcript. The DNA fragment (labeled at the non-template strand) containing the AdML promoter was incubated with wild type (lane 1-3) or mutant (lane 4-6) TFIIH and other basal factors. The sensitivity to potassium permanganate modification was subsequently determined in the absence of nucleotides (lane 1 and 4), or in the presence of ATP (lane 2 and 5), or ATP, CTP, UTP, and 3'-OMeGTP (lane 3 and 6). Indicated are the positions of thymidine residues with respect to the transcriptional start site as determined with a G+A ladder (not shown). The bar indicates the open region of the non-template strand.

Earlier stages of open complex formation, in which a region upstream of the start site is still open, were analyzed using template DNA that allows stalling of the polymerase after the formation of a 4-nucleotide transcript. Again, no difference could be discerned when wild type or mutant TFIIH were used (data not shown). Together, these comparative analyses between wild type and mutant TFIIH suggest that the XPD helicase activity is dispensible for transcription initiation by RNA polymerase II *in vitro*.

TFIIH containing inactive XPD ATPase subunit is able to support damage-dependent DNA synthesis

To analyze the requirement for ATP hydrolysis by the XPD subunit of TFIIH during NER, we carried out *in vitro* NER reactions. A WCE was prepared from CHO UV5 repair-deficient cells and incubated in the absence or presence of wild type and K48R mutant TFIIH complexes, AAF-damaged DNA and undamaged control plasmid, and radiolabeled dATP and nucleotides. After incubation, plasmid DNA was isolated, linearized and subjected to agarose gel electrophoresis. Ethidium

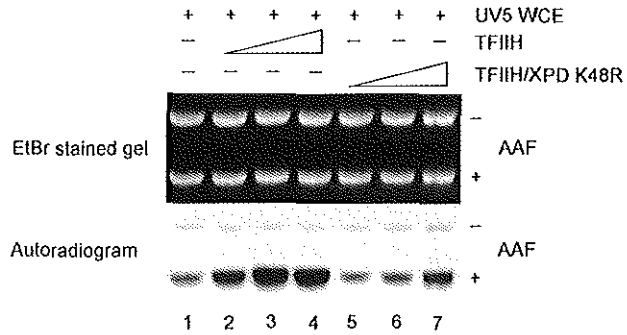

Figure 5. K48R XPD complex stimulates damage-dependent DNA synthesis *in vitro*. Wild type (1, 2, 4 µl, lane 2-4, respectively) and K48R XPD complex (1, 2, 4 µl, lane 5-7, respectively) were assayed for complementing activity of CHO UV5 (XP-D) extracts (125 µg, lane 1-8). The positions of the undamaged (-) and AAF-damaged (+) plasmids are indicated. *Upper panel*, ethidium bromide stained agarose gel. *Lower panel*, autoradiogram of the same gel.

bromide staining indicated that equal amounts of DNA were recovered from each reaction (Fig. 5, upper panel). The corresponding autoradiogram (Fig. 5, lower panel) shows that only slightly more radiolabel is incorporated in the damaged plasmid incubated with UV5 WCE alone compared with the non-damaged control, while increasing amounts of wild type TFIIH are able to stimulate DNA repair synthesis. Surprisingly, TFIIH containing K48R XPD was also able to stimulate DNA synthesis specifically in the plasmid containing AAF-damage, although to a lower level as compared to wild type. From this experiment we estimate that the incorporation of nucleotides by DNA repair synthesis by the mutant complex is about 25% of the wild type fraction. These findings suggested that the K48R XPD mutation might not completely impair the NER function of TFIIH as assessed in the *in vitro* NER reaction. Therefore, we analyzed in more detail the formation of NER reaction-intermediates.

The formation of incisions around a DNA-damage is defective in the presence of TFIIH with K48R mutant XPD

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 using a specific and sensitive method which detects the 24-32 nt platinated oligonucleotides released by the NER system. We assessed the activity of the mutant TFIIH in NER using a fully reconstituted system (Fig. 6*A*) and by complementation of two TFIIH-defective cell lines (Fig. 6*B*). For the latter purpose WCE were prepared from two human repair-deficient cell lines, XP-B (GM2252) and XP-D (GM2485). 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 from HeLa cells (Fig. 6*A*). However, reaction mixtures containing TFIIH with mutant K48R XPD subunit showed no detectable release of damaged oligonucleotide (Fig. 6*A*). We estimate from other experiments that the sensitivity of the assay would detect dual incision activity as low as 5% of the wild type level. Mutant K48R XPD-TFIIH complex was, however, able to weakly complement an extract derived from XP-B cells (Fig. 6*B*). Correction of the incision defect by the mutant complex is 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, when wild type or mutant complex was added to extracts from XP-D cells, only the wild type was able to correct the incision defect (Fig. 6*B*).

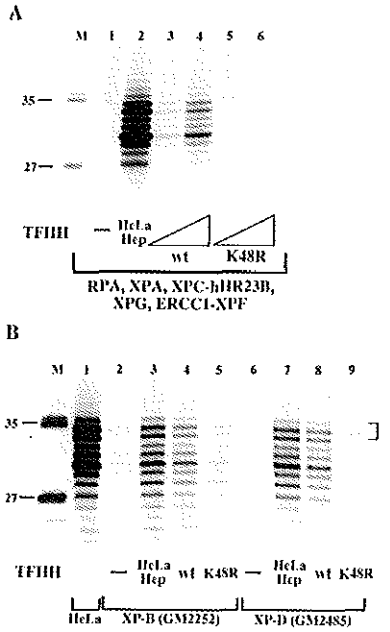

Figure 6. Dual incision activity of wild type and K48R mutant XPD *in vitro*. *A*, Reconstitution of nucleotide excision repair *in vitro* with purified components; reactions included either HeLa TFIIH (1.5 µl Hep fr. IV) or wild type or K48R mutant TFIIH (1.5 µl and 3 µl) as indicated; lane M, size markers (pBR322-*Msp* I digest). *B*, Complementation of XP-B and XP-D defective cell extracts with wild type or K48R mutant TFIIH; each reaction contained 20 µg of whole cell extract protein and was complemented with 1 µl HeLa TFIIH or 2 µl wild type XPD or K48R mutant complex as indicated; lane M, size markers; the weak bracketed bands (lane 9) occasionally arise from non-specific end-labelling of the complementary oligonucleotide used in the detection method.

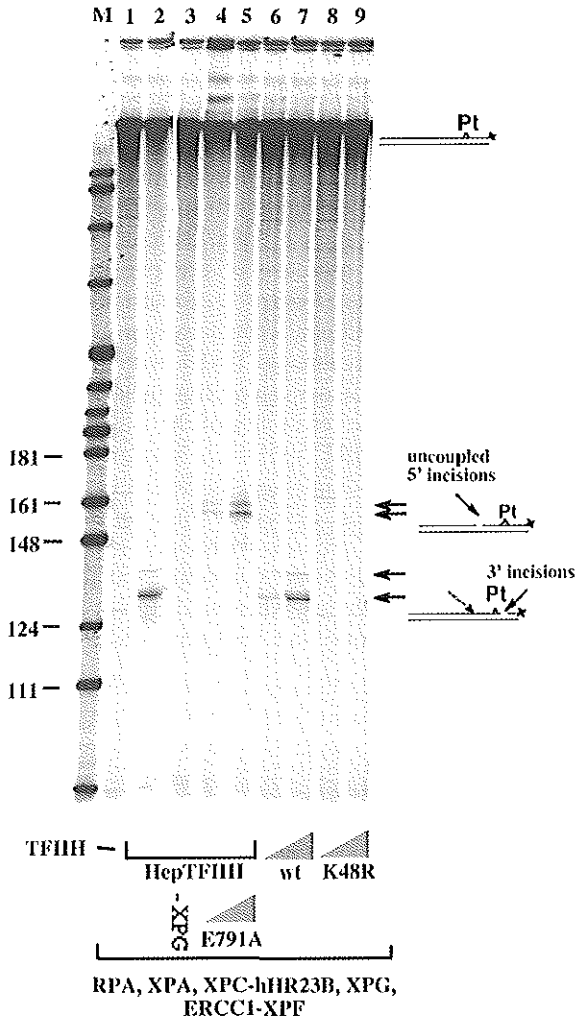

Figure 7. Activity of TFIIH with wild type or K48R mutant XPD around a single Pt-GTG lesion. Reconstitution of nucleotide excision repair *in vitro* using an assay that reveals specific sites of enzymatic incision on the 3' or 5' sides of the platinum lesion. The (Pt-GTG) duplex substrate is radiolabelled on the damaged strand 140 nt 3'of the adduct. Lane M, size markers (pBR322-*Msp* I digest). Reactions contained purified components except as indicated: lane 1 contains no TFIIH; lanes 2-5 contain HeLa TFIIH (1.5 µl Hep fr. IV), lane 3 contains no XPG protein; lane 4 and 5 contain 0.75 and 1.5 ng, respectively, of E791A mutant XPG (42); lanes 6-9 contain the indicated TFIIH complexes containing wild type or K48R mutant XPD subunit; uncoupled 5' incision and 3' incision products are indicated.

To establish whether mutant TFIIH allows the formation of uncoupled 3' or 5' incisions, the single lesion DNA substrate was labeled at the 3' end with respect to the lesion and incubated with purified components. This assay detects all 3' incisions, arising during the dual incision reaction or as uncoupled 3' or 5' incisions (see diagrams at the right of Fig. 7). In the presence of wild type TFIIH, 3' incisions were readily formed (during the dual incision reaction, Fig. 7, lane 6,7). However, no 3' nor 5' incisions were detected when the reactions were performed with TFIIH containing mutant XPD subunit (Fig. 7, lane 8,9). As a positive control, uncoupled 5' incisions were efficiently placed in the presence of E791A mutant XPG protein (Fig. 7, lane 4, 5) (42). Taken together, these data indicate that the efficient formation of both incisions around a DNA lesion depends on ATP hydrolysis by the XPD subunit of TFIIH.

K48R XPD cDNA stimulates unscheduled DNA synthesis but does not confer UV-resistance to XP-D cells

The physiological relevance of the above findings for NER was investigated. Therefore, the cDNAs encoding the wild type and K48R protein were injected into nuclei of living primary human XP-D fibroblasts. Upon injection of wild type cDNA, unscheduled DNA synthesis (UDS) was stimulated up to the level of wild type cells (Table 1). Interestingly, injection of mutant cDNA encoding the K48R protein stimulated the residual UDS of the XP6BE XP-D fibroblast to about 50% of wild type levels. The *in vivo* observed increase in UDS from roughly correlates with the increase in DNA synthesis measured in the *in vitro* assay (see Fig. 5). Similar results were obtained using a different XP-D cell line (XP1BR, data not shown). We failed to detect any dominant effect exerted by the mutant protein on either RNA synthesis or UDS after injection of wild type fibroblasts (data not shown).

Table 1. Microinjection of cDNA encoding XPD(K48R) protein stimulates unscheduled DNA synthesis in living XP-D fibroblasts.

cell type	cDNA injected	UDS (grains/nucleus)[†]	percentage
C5RO (wild type)	non-injected	65.8 ± 1.8	100 ± 2.7
XP6BE (XP-D)	non-injected	11.5 ± 0.6	17.5 ± 5.0
	XPD	66.8 ± 2.4	101.5 ± 3.5
	XPD(K48R)	31.0 ± 1.7	47.2 ± 5.4

[†] Autoradiographic grains above the nuclei of at least 50 cells were counted.

To investigate whether the observed increased UDS levels represents a low-level of DNA repair, the cDNA encoding wild type and K48R protein were transfected into XPD-deficient CHO and human cells and UV-survival was analyzed. Fig. 8*A* shows an immunoblot of CHO UV5 cell lysates obtained from selected clones expressing wild type and mutant cDNA. Using a monoclonal antibody raised against human XPD, a specific band with equal intensity

corresponding to the expected molecular mass of XPD is observed in lanes containing lysates from UV5 cells transfected with wild type or mutant human cDNA. This band is not observed in lanes containing lysate from UV5 cells or empty-vector transfected cells, indicating that UV5 cells express the human cDNA to the same level, and that the monoclonal antibody does not cross-react with the mutant Chinese hamster XPD protein. The UV-survival experiment (Fig. 8*B*) shows that the wild type human cDNA confers UV-resistance to UV5 cells, up to the level of the parental cell line AA8. However, the mutant cDNA encoding K48R XPD failed to confer significant UV-resistance as compared to empty-vector transfected UV5 cells as noted before (43).

To further analyze whether the observed increase of UDS represents NER events and to exclude that the inability of the human mutant cDNA to confer UV-resistance to CHO XPD-deficient cells is due to a subtle cross-species complementation defect, a similar experiment was performed using human XP-D cells. Expression of the cDNA encoding wild type and K48R mutant proteins was analyzed by immunoblotting (Fig. 8*C*). A specific band was only observed in lysates containing the wild type and K48R proteins in approximately equal amounts when compared with a lysate derived from immortalized wild type cells. Interestingly, the antibody did not recognize the endogenous mutant protein present in the XP-D cells. As in the case of transfection of CHO cells, the cDNA encoding K48R XPD failed to confer UV-resistance to human cells, while the cDNA encoding the wild type protein did, almost to the level of wild type control cells (Fig. 8*D*). However, the cDNA encoding K48R XPD was able to confer increased UDS to human XP-D cells (Fig. 8*E*), up to approximately 50% of the wild type levels, in agreement with the microinjection experiments. These results clearly indicate that cellular UV-resistance and efficient NER requires XPD helicase activity, although significant damage-dependent DNA synthesis is supported by TFIIH containing an enzymatically inactive XPD subunit.

DISCUSSION

Role of XPD in transcription

In this paper, five criteria are presented which indicate directly that mammalian XPD activity is not required for RNA polymerase II-mediated basal transcription: (i) TFIIH containing an inactive XPD helicase is able to support basal transcription in a reconstituted system with highly purified components; (ii) neither enzymatic formation of tri(di)-nucleotide transcripts nor productive formation of 15-nucleotide transcripts are significantly affected; (iii) open complex formation after synthesis of a 15-nucleotide transcript is nearly identical in the presence of either wild type or K48R mutant XPD; and (iv) microinjection of XPD cDNA encoding K48R protein

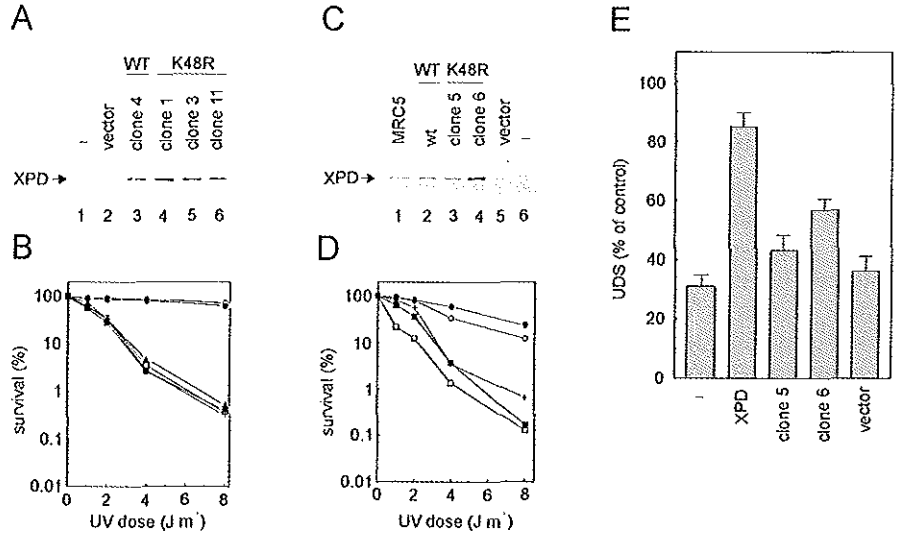

Figure 8. cDNA encoding K48R XPD protein does not confer UV-resistance to CHO or human cells, but stimulates unscheduled DNA synthesis in human cells. *A*, Immunoblot analysis using anti-XPD monoclonal antibodies 2F6. Lysates were obtained from CHO UV5 cells (lane 1), or CHO cells transfected with empty vector (lane 2), cDNA encoding wild type XPD (clone 4, lane 3), or cDNA encoding K48R XPD (clone 1, 3, and 11, lane 4, 5, and 6, respectively). *B*, UV-survival analysis of UV5 CHO cells transfected with empty vector (+), cDNA encoding wild type XPD (o), or cDNA encoding K48R XPD (□, clone 1; ■, clone 3; ▲, clone 11), and the parental cell line AA8 (•). *C*, Immunoblot analysis of lysates of human MRC5 immortalised wild type fibroblasts (lane 1), HD2 cells transfected with cDNA encoding wild type XPD (lane 2), or cDNA encoding K48R XPD (clone 5 and 6, lane 3 and 4), empty vector transfected (lane 5) and untransfected HD2 cells (lane 6). *D*, UV-survival analysis of HD2 human cells transfected with empty vector (+), cDNA encoding wild type XPD (o), or cDNA encoding K48R XPD (■, clone 5; □, clone 6), and a wild type cell line MRC5 (•). *E*, Analysis of unscheduled DNA synthesis (UDS) of HD2 transfected cells. Levels of UDS are represented as values relative to those of wild type MRC5 cells. Error bars indicate the standard error of the mean.

does not interfere with RNA synthesis in living fibroblasts and therefore does not seem to have dominant-negative effects. This is in striking contrast to the corresponding mutation in the XPB helicase subunit, which was found to paralyse transcription and DNA repair in a dominant fashion (32). (v) Stable transformants carrying the K48R mutant protein in TFIIH are viable, suggesting that the mutation does not impair transcription. These findings are in agreement with and further extend observations made in *S. cerevisiae* (44,45) and studies using recombinant TFIIH (46).

In contrast to the requirement for the enzymatic activity of XPD, it is evident that the physical presence of the protein is required for transcription and viability (47,48). Mutations in XPD that interfere with transcription should therefore

compromise other functions of the protein, perhaps in addition to a defect in the ATPase activity, such as interactions with other transcription proteins or stability of TFIIH *in vivo* (24).

Role of XPD in NER

The findings in this paper show that if a mutation inactivates only the enzymatic activity without significantly altering the conformation of the XPD protein, then this results in considerable damage-dependent DNA synthesis. However, the observed synthesis does not reflect effective DNA repair, because a cDNA encoding the mutant protein is unable to confer significant UV-resistance to XP-D cells and TFIIH containing mutant XPD is unable to efficiently place either 5' or 3' incisions at defined positions around a DNA lesion. It is possible that the K48R XPD-TFIIH can still carry out 5% or less of normal incision, below our level of detectability, but this does not seem sufficient to account for the 25-50% UDS seen in XP-D cells. Nevertheless, the existence of substantial damage-dependent DNA synthesis strongly suggests that some damage-dependent DNA strand-cleavage is occurring, which may not depend on the NER endonucleases.

Mechanisms of unwinding by TFIIH

In the oligonucleotide-displacement assay, no activity could be observed with TFIIH containing K48R XPD subunit. This is in agreement with the observation that the contribution of the XPB helicase is minor compared to that of XPD in this type of assay (46,49). However, opening of a promoter region in transcription initiation is supported by the XPD mutant form of TFIIH within the limits of the assay in a fashion indistinguishable from the wild type complex. Obviously, in these two assays different activities associated with TFIIH are measured. This is also reflected in the apparent K_m for ATP in the two reactions: in transcription, the K_m for (d)ATP has been determined to be 0.25-2 μM (50-52), while the apparent K_m for ATP in the oligo-displacement reaction is ≈100 fold higher (150-200 μM (18); F.C.P. Holstege and H.Th.M.T., unpublished results). A reason for these large differences may be that in transcription TFIIH is differently positioned in the presence of other factors and opening of only 6 bp may already be sufficient (12). Since the XPB helicase is absolutely required for transcription *in vivo* (32,53), it is possible that the limited opening in transcription is predominantly due to XPB activity. The differential requirement for XPD activity in transcription and NER may originate from the fact that in the NER reaction the initial unwinding by TFIIH must be more extended, demanding both XPB and XPD activities, before other factors can participate in opening and stabilize the unwound structure.

Implications for human disease

Mutations in XPD can result in XP, XP/CS, and TTD syndromes, while mutations in XPB, which are much more infrequent, are associated with XP/CS and TTD. It has been proposed that the additional clinical manifestations seen in XP/CS and TTD are due to a defect in the transcription function of TFIIH. The fact that only very few XPB mutations are identified as compared to XPD, correlates with the notion that XPB plays a more important role in transcription initiation. Likewise, the observation that some XPD mutations give rise to XP, correlates with the data presented here that the function of XPD in NER and transcription can be separated. If the enzymatic activity of XPD is indeed dispensable for transcription, then this would predict that an allele encoding the K48R mutation would result in an XP-like phenotype. No such mutation has been identified yet, although a mutation in the Walker type A domain has been described (G47R) causing an XP phenotype (54) without clear symptoms associated with a transcriptional defect. To further test the above hypothesis and to establish the significance of the XPD helicase activity for transcription *in vivo*, it would be of interest to generate a mouse model by introducing the K48R point mutation in the murine XPD gene by homologous recombination.

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CHAPTER 4

ATP-dependent Release of Inhibition of DNA Repair Endonucleases XPG and ERCC1-XPF by TFIIH

Submitted for publication

ATP-DEPENDENT RELEASE OF INHIBITION OF DNA REPAIR ENDONUCLEASES XPG AND ERCC1-XPF BY TFIIH

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The multisubunit basal transcription factor IIH (TFIIH) has a dual involvement in nucleotide excision repair (NER) of a variety of DNA lesions, including UV-induced photoproducts, and RNA polymerase II transcription. In both processes, TFIIH is implicated with local DNA unwinding, which is attributed to its two helicase subunits, XPB and XPD. To further define the role of TFIIH in NER, functional interactions between TFIIH and other DNA repair proteins were analyzed. We show that the TFIIH-associated ATPase activity is stimulated both by XPA and the XPC-HR23B complex. However, while XPA promotes the ATPase activity specifically in the presence of damaged-DNA, stimulation by XPC-HR23B is lesion independent. Secondly, we demonstrate that the TFIIH-associated kinase activity phosphorylates XPG and XPA *in vitro*. Finally, using model substrates that mimic NER intermediates, we reveal that TFIIH inhibits the structure-specific endonuclease activities of both XPG and ERCC1-XPF, responsible for the 3' and 5' incision in NER, respectively. The inhibition occurs in the absence of ATP, and is reversed upon addition of ATP. These results point towards additional roles for TFIIH and ATP during NER distinct from a requirement for DNA unwinding in the regulation of the endonuclease activities of XPG and ERCC1-XPF.

INTRODUCTION

Nucleotide excision repair (NER) is one of the major DNA repair pathways essential for the removal of UV-induced photoproducts and bulky adducts from DNA (see Ref.(1-3) for comprehensive overviews of DNA repair). The basic mechanism is conserved from prokaryotic *E. coli* to human cells and can be divided into two major stages: (i) recognition and dual incision of the damaged strand on both sides of the DNA lesion, and (ii) gap-filling repair DNA synthesis by the replication machinery. In humans, defects in factors involved in the dual incision step are associated with three distinct inherited disorders: xeroderma pigmentosum (XP), a combined form of XP and Cockayne's syndrome, and a photosensitive form of trichothiodystrophy. These syndromes are characterized by a pleiotropic phenotype including (hyper)sensitivity to sun(UV)-light and extensive clinical and genetic heterogeneity (reviewed in (4)).

Six human protein factors are required for recognition and dual incision *in vitro* (5,6). The XPC-HR23B complex binds with high specificity to DNA damage and represents the initial recognition factor able to recruit the remainder of the NER apparatus (7). Three additional factors are required for the subsequent ATP-dependent local melting of the DNA helix around the lesion (8-10): basal transcription factor IIH (TFIIH), which has an additional, essential cellular function

in transcription by RNA polymerase II (see ref. (11,12) for further details); XPA, which preferentially binds to DNA containing lesions as well as single-stranded DNA (13); and the single-stranded DNA binding protein RPA, which most likely binds to the undamaged strand (14). The locally unwound region provides a substrate for the structure-specific endonucleases XPG and ERCC1-XPF, which incise the damaged strand at the 3' and 5' side, respectively (15,16).

TFIIH possesses several enzymatic functions: the DNA-dependent ATPase and DNA helicase activities are attributed to both of the largest subunits, XPB and XPD (17,18). CDK7 was identified as the kinase catalytic subunit, which is able to phosphorylate the C-terminal domain of the largest subunit of RNA polymerase II (19,20). In NER, TFIIH has been implicated with a function in DNA unwinding by virtue of its helicase subunits (9). However, TFIIH may have additional roles in the dual incision reaction. Full DNA opening around a lesion is observed in a XP-B extract derived from XP11BE cells with a mutation that affects the C-terminal 40 aminoacids of the XPB subunit of TFIIH (9). Despite complete opening, however, the formation of the 5' incision is defective (9). Furthermore, NER substrates with a DNA lesion located in a pre-melted region still require TFIIH for dual incision (10).

In this report, we have analyzed how the enzymatic activities of TFIIH are influenced by other NER factors and *vice versa* and identified a novel function for TFIIH and ATP distinct from DNA unwinding.

MATERIALS AND METHODS

Purification of NER factors

Recombinant mouse XPA and human RPA were both purified from *E.coli* strain BL21(DE3)pLysS using plasmids pET-8c-XPA (kindly provided by Drs. C.F. van Kreyl and H. van Steeg) and p11d-tRPA (a generous gift of Dr. M.S. Wold) exactly as described previously (7,21). Human XPC-HR23B complex was reconstituted from recombinant XPC, purified from baculovirus-infected insect cells, and HR23B purified from *E.coli*, as described (22,23). Human XPG endonuclease was obtained from insect cells infected with recombinant baculovirus (generously provided by Dr. R.D. Wood) using a procedure described before (14). Recombinant human ERCC1-XPF complex was partially purified from *E.coli* according to a procedure that will be described in detail elsewhere. Highly purified TFIIH was obtained either from human fibroblasts expressing epitope-tagged XPB (24), or from HeLa cells using classical chromatography (25). TFIIH containing fractions from the last steps of either purification were used and gave identical results.

Analysis of ATPase activities

Stimulation of the TFIIH-associated ATPase activity was analyzed in 10-µl reaction mixtures (24,26). Reaction mixtures contained approximately 100 ng TFIIH, 50 ng XPA or XPC-HR23B complex, and 100 ng DNA as indicated. Control reactions contained approximately 100 ng UvrA, and 75 ng UvrB (generously obtained from Dr. N. Goosen). Reactions were terminated by the addition of 5 µl 0.5 M EDTA and 0.2 µl of each reaction was analyzed by thin-layer chromatography on polyethylenimine-cellulose plates (Merck) (24).

Phosphorylation of NER factors

Phosphorylation of NER factors by TFIIH was analyzed in 15-µl reaction mixtures as described (24). Reactions contained approximately 60-70 ng TFIIH, and 50-125 ng XPG, or 50 ng XPA or XPC-HR23B complex. To analyse nucleotide specificity, 100 µM (>2000-fold molar excess) of the indicated unlabeled (ribo)nucleotide was added as competitor cofactor.

Analysis of nuclease activity

Nuclease reactions were carried out in 15-µl volumes in buffer containing 0.75 mM $MnCl_2$ (14). As a model substrate, a hairpin of 22 bp with either a 3'-end or 5'-end protruding-$d(T)_{28}$ arm was used as described previously (14). Per reaction, 125 ng purified XPG endonuclease or approximately 1.0 µg protein fraction of partially purified recombinant ERCC1-XPF complex was added to 1.5 ng labeled substrate. Inhibition by TFIIH (approximately 100 ng) was reversed in the presence of 1 mM ATP. Availability of the restriction enzyme site in the duplex region was analyzed by addition of *Hae*III (10 Units; Boeringer Mannheim) to a pre-assembled reaction mixture.

RESULTS

Stimulation of the TFIIH-associated ATPase activity by XPA and XPC-HR23B

Hydrolysis of the β-γ bond of ATP by TFIIH is an essential step during NER and at least part of the energy is required for the formation of a melted region around a

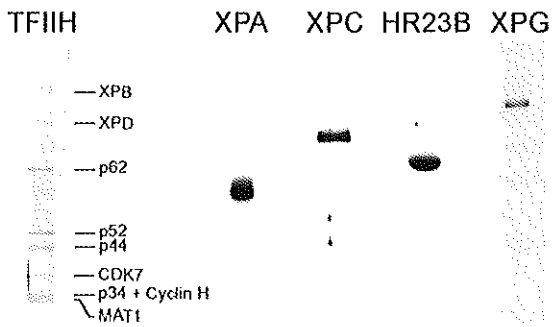

Figure 1. Purified protein factors used in this study. SDS-polyacrylamide gels containing TFIIH stained with silver nitrate; XPA, XPC, and HR23B, used to reconstitute recombinant XPC-HR23B complex as described in "Materials and Methods", and XPG stained with Coomassie blue. The individual subunits of the multisubunit factor TFIIH are indicated.

DNA damage (8). In addition to TFIIH, three protein factors are minimally required for local DNA unwinding: XPA, XPC-HR23B, and RPA (10), which may stimulate the DNA-dependent ATPase activity of TFIIH. To find evidence for this hypothesis and identify individual factors involved, the effect of these proteins on the ATPase activity of TFIIH was analyzed using purified proteins (Fig. 1). As is shown (Fig. 2*A, B*), the TFIIH-associated ATPase was markedly stimulated in the presence of plasmid DNA, which occurred to the same extent when *N*-acetoxy-AAF-damaged plasmid DNA was added to the reaction (Fig. 2*A*, lane 4-6). However, while purified XPA alone did not have any influence on ATP hydrolysis (Fig. 2*A*, lane 7-9), the TFIIH-associated ATPase activity was strongly increased in the presence of both XPA and damaged-DNA (Fig. 2*A*, lane 10-12). Interestingly, XPC-HR23B, which did not hydrolyse ATP (Fig. 2*A*, lane 13-15), was also able to stimulate the TFIIH-associated ATPase. However, although the stimulation by XPC-HR23B depended on the presence of DNA, no additional specificity for DNA lesions was present (Fig. 2*A*, lane 16-18). This effect was also observed when both XPA and XPC were added, indicating that an interaction between XPC and TFIIH was preferred under the conditions used. Addition of RPA did not result in increased ATPase activity of TFIIH, nor did it significantly increase the stimulation by XPA (data not shown).

In *E. coli*, NER requires only three proteins for recognition and dual incision, including UvrA and UvrB, which have no homology at the aminoacid level with eukaryotic NER proteins. Both XPA and XPC-HR23B did not stimulate the ATPase activity of the prokaryotic UvrA protein (Fig. 2*C*, compare lane 7-9 and 10-12 with lane 4-6), providing further evidence for the specificity of the stimulation of the TFIIH-associated ATPase activity by XPA and XPC-HR23B. In contrast, UvrB did stimulate the ATPase activity of UvrA, indicating that the protein was responsive to a specific interaction (Fig. 2*C*, lane 13-15).

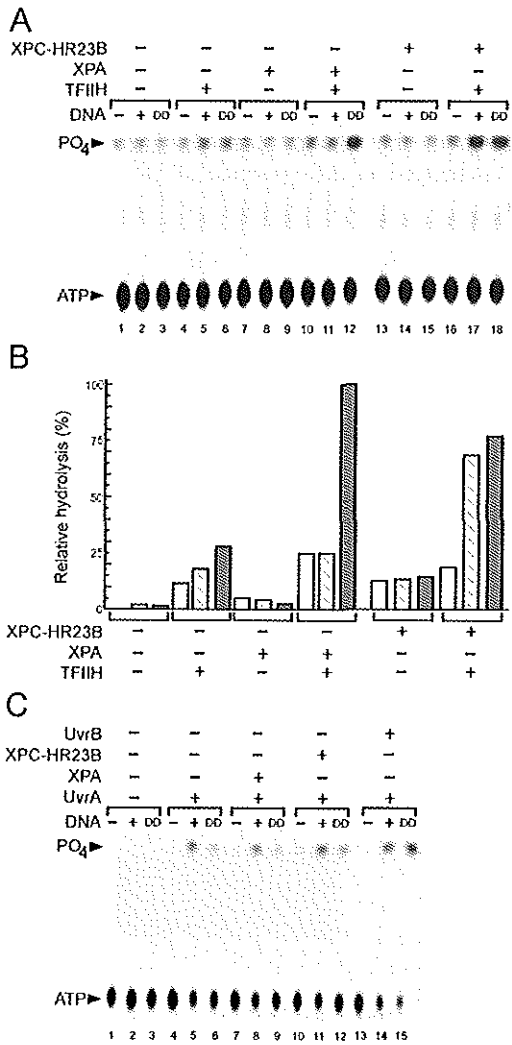

Figure 2. Stimulation of the TFIIH-associated ATPase activity by XPA and XPC-hHR23B. *A*, Autoradiograms showing the effect of XPA and XPC-HR23B on the TFIIH-associated ATPase. Free phosphate was separated from ATP by thin-layer chromatography. Reactions contained factors as indicated in the figure and were carried out in the absence of DNA (-), in the presence of plasmid DNA (+), or in the presence of AAF-damaged DNA (DD). The stimulation of the ATPase of TFIIH by XPA in the absence of DNA was not consistently observed. *B*, Relative hydrolysis of the ATPase activities shown in *A*. The values obtained in the absence of any protein and DNA or in the presence of XPA and TFIIH were arbitrarily set at 0 and 100%, respectively. Open bars represent reactions in the absence of DNA, hatched and filled bars represent the presence of plasmid DNA and AAF-containing plasmid DNA, respectively. *C*, Autoradiograms showing no effect of XPA and XPC-HR23B on ATP-hydrolysis by UvrA. Reactions contained factors as indicated in the figure and were carried out in the absence of DNA (-), in the presence of plasmid DNA (+), or in the presence of AAF-damaged plasmid DNA (DD). Released phosphate was separated from ATP by thin-layer chromatography. Indicated are the positions of free phosphate and ATP.

TFIIH phosphorylates NER factors in vitro

Previous studies indicated that reversible phosphorylation is important for optimal dual incision *in vitro* (27). However, a specific target for phosphorylation is not identified (27,28). Because TFIIH is the only known NER factor with an associated kinase activity, TFIIH-mediated phosphorylation may be important in the repair reaction. Therefore, we analyzed whether TFIIH was able to phosphorylate NER proteins involved in the dual incision stage using purified factors (Fig. 1). As shown

in Fig. 3*A*, TFIIH did not undergo significant auto-phosphorylation under the conditions used. However, XPG was phosphorylated efficiently in the presence of TFIIH. Weak phosphorylation of XPA and no or barely detectable phosphorylation was observed of the other NER factors as indicated (Fig. 3*A*), or of RPA (data not shown), supporting substrate specificity in the kinase reaction. The observed phosphorylation specificity appeared unchanged in the presence of DNA with or without AAF-lesions (data not shown).

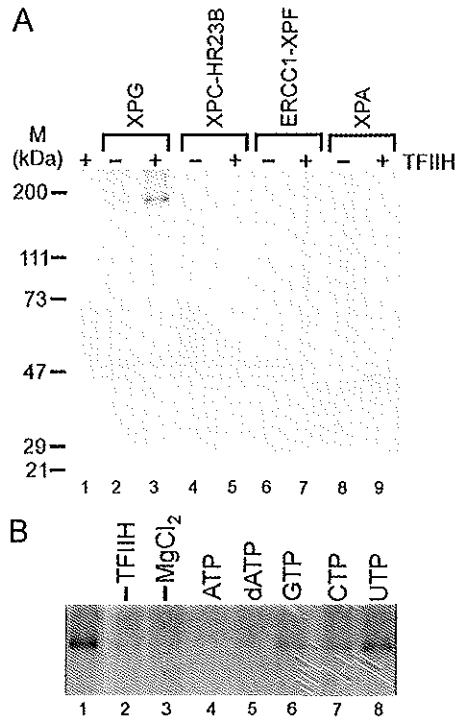

Figure 3. Phosphorylation of XPG by the TFIIH-associated kinase activity. *A*, Autoradiogram of SDS-PAGE analysis. Reactions contained factors as indicated either in the absence (-) or presence (+) of TFIIH. The positions of the molecular weight markers are indicated. *B*, Autoradiogram of SDS-PAGE indicating cofactor specificity of XPG phosphorylation by TFIIH. A kinase reaction was carried out as described in "Materials and Methods" (lane 1) or in the absence of TFIIH or $MgCl_2$ (lane 2 and 3, repectively) or in the presence of unlabeled competitor nucleotide (100 μM of each nucleotide as indicated above lane 4-8).

To determine further specificity in the XPG phosphorylation reaction, the cofactor requirements were investigated. Efficient phosphorylation of XPG by TFIIH was dependent on Mg^{2+} (Fig. 3*B*), which could be substituted by Mn^{2+} (data not shown). The phosphorylation of XPG by radiolabeled ATP was completely inhibited by the addition of >2000-fold excess unlabeled (d)ATP, but not by addition of GTP, CTP or UTP. A similar nucleotide cofactor specificity for the TFIIH-associated protein kinase is observed with RNA polymerase II as a substrate (26).

ATP-reversible inhibition by TFIIH of the XPG endonuclease

After open complex formation, the first stage of the NER reaction is completed by dual incision of the damaged strand on both sides of the lesion. Whether TFIIH has a direct effect on the endonucleolytic activities of XPG and ERCC1-XPF was investigated using model substrates that mimic NER intermediates. These substrates consist of oligonucleotides containing partially self-complementary regions resulting in DNA duplexes of 22 bp and either a 5'-end or 3'-end protruding single-stranded arm of 28 thymidine residues that will minimize the formation of secondary structures. These oligonucleotides are recognized and incised at defined sites by both NER endonucleases and allow to study the effect of a single NER factor on XPG and ERCC1-XPF activity (14).

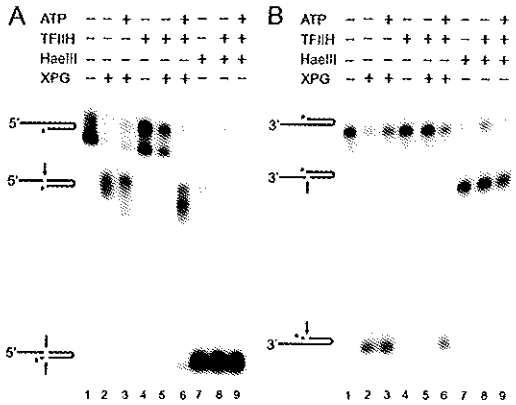

Figure 4. Autoradiogram of nuclease assay indicating ATP-reversible inhibition of XPG endonuclease activity by TFIIH. *A*, Reactions containing 5'-end protruding-d$(T)_{28}$ substrate and factors as indicated above the lanes. The DNA substrate used displayed a heterogeneous migration pattern (lane 1), presumably due to melting and reannealing of the duplex part of the oligonucleotide during electrophoresis. The smearing of the incision product may also be due to migration conditions and/or at least in part to a 5' to 3' exonuclease activity associated with XPG (34). *B*, Reactions containing 3'-end protruding-d$(T)_{28}$ substrate and factors as indicated above the lanes. Note that, independent of the presence of TFIIH and/or ATP, *Hae*III was hardly able to digest the non-protruding strand of the 3'-end protruding substrate, as observed before (14).

When XPG was incubated with a DNA duplex containing a 5'-end protruding single-stranded arm, a specific incision was observed in the duplex region in the strand that continues as the 5'-end protruding arm (Fig. 4*A*, compare lanes 1 and 2). Addition of ATP had no or little effect on the reaction efficiency, while TFIIH alone did not contain any detectable nuclease activity under the conditions used (Fig. 4*A*, lanes 3 and 4, respectively). Unexpectedly, when TFIIH was added to the XPG reaction mixture in the absence of ATP, a complete inhibition of XPG nuclease activity was observed (Fig. 4*A*, lane 5). Surprisingly, addition of ATP to the reaction containing TFIIH and XPG fully reversed the inhibitory effect of TFIIH (Fig 4*A*, lane 6). In these reaction mixtures, the DNA substrate was present in sub-stoichiometric amounts compared to both XPG and TFIIH. However, because XPG was present in a large excess over TFIIH (at least 5-fold), the reaction stoichiometry strongly suggests that inhibition in the absence of ATP is mediated by binding of TFIIH to DNA, thereby blocking XPG nuclease activity.

The inhibition of XPG activity by TFIIH was not due to aspecific blocking of the DNA duplex/single-stranded junction, as was measured by availability of a restriction enzyme site (GGCC; *Hae*III) at the last four base-pairs of the duplex region. When *Hae*III was added to the reaction mixture containing the DNA substrate and TFIIH either in the presence or absence of ATP restriction enzyme digestion of the DNA was unaffected (Fig. 4*A*, lanes 7-9), which in addition supports the idea that no stable DNA unwinding occurs in the presence of TFIIH and ATP.

The inhibition by TFIIH of the endonucleolytic activity of XPG did not depend on the polarity of the DNA duplex/single-stranded junction. Using a substrate containing an identical duplex region, but with a 3'-end $d(T)_{28}$-protruding arm, similar results were obtained (Fig 4*B*). XPG efficiently incised the duplex region in the strand opposite of the 3'-end protruding arm (Fig. 4*B*, lane 2). TFIIH completely inhibited cleavage by XPG of this substrate (Fig. 4*B*, lane 5), which was reversed by the addition of ATP to the reaction mixture (Fig. 4*B*, lane 6). TFIIH-mediated inhibition of XPG activity on this substrate was not due to aspecific blocking of the duplex/single-stranded junction, as measured by *Hae*III restriction enzyme digestion (Fig. 4*B*, lane 7-9) consistent with the notion that aspecific blocking and/or ATP-dependent stable DNA unwinding did not occur on this substrate either.

Effect of TFIIH and ATP on ERCC1-XPF endonuclease activity

The observed ATP-reversible inhibition of XPG activity by TFIIH prompted to investigate whether this effect was specific for XPG, or general for both NER endonucleases. Therefore, partially purified recombinant ERCC1-XPF was incubated with a 5'-end protruding-$d(T)_{28}$ DNA substrate (Fig. 5*A*, lane 2) or with the 3'-end protruding-$d(T)_{28}$ substrate (Fig. 5*B*, lane 2). As indicated, ERCC1-XPF was able to cut these substrates at specific sites and with opposite polarities as compared to the XPG endonuclease (compare Fig. 4*A* and 5*A*, and Fig. 4*B* and 5*B*, lanes 2). Addition of ATP to the reaction mixtures containing ERCC1-XPF inhibited the reactions considerably (Fig. 5*A* and *B*, lanes 3). This effect might amongst others be due to sensitivity of ERCC1-XPF for ATP or the increased ionic strength of the added ATP solution. Regardless of this effect, however, TFIIH was able to completely inhibit ERCC1-XPF activity even in the absence of ATP (Fig. 5*A* and *B*, lanes 5), while TFIIH alone did not have any effect on the DNA substrates (Fig. 5*A* and *B*, lanes 4). Interestingly, despite its inhibitory effect and the presence of TFIIH, addition of ATP to reaction mixtures containing TFIIH and ERCC1-XPF partially restored nuclease activity compared to the level of the control reaction containing ATP and ERCC1-XPF only (Fig. 5*A*, compare lanes 6 and 3, and Fig. 5*B*, compare lanes 6 and 3).

These findings suggest that ATP-reversible inhibition by TFIIH is a general mechanism for both NER endonucleases. Because the incision sites of XPG and ERCC1-XPF and the availability of the *Hae*III restriction enzyme site appeared

unchanged in the presence of both TFIIH and ATP, stable unwinding of the DNA duplex region does not occur to significant extent under the conditions used. Because ERCC1-XPF is not phosphorylated by TFIIH *in vitro*, at least in this case it is likely that the ATP-reversed inhibition is mediated by the DNA-dependent ATPase/helicase subunits of TFIIH *via* a mechanism distinct from DNA unwinding.

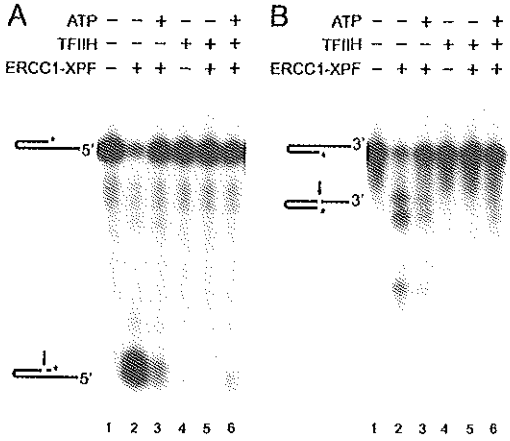

Figure 5. Autoradiogram of nuclease assay showing ATP-reversible inhibition of ERCC1-XPF endonuclease activity by TFIIH. *A*, Reactions containing 5'-end protruding-d(T)$_{28}$ substrate and factors as indicated above the lanes. *B*, Reactions containing 3'-end protruding-d(T)$_{28}$ substrate and factors as indicated above the lanes. Note that addition of ATP only (*A*, *B*; lanes 3) inhibited the nuclease reaction considerably.

DISCUSSION

Besides an essential cellular transcription factor, TFIIH is a core constituent of NER, involved in helix unwinding. Many protein interactions described between NER factors appear not stable and may represent transient interactions (24,29). In this report, the functional effect of repair factors on TFIIH activity and *vice versa* was analyzed.

Although XPC-HR23B does not stably associate with TFIIH (24), it stimulates the ATPase activity of the latter complex, which may be due to an increased affinity of TFIIH for DNA in the presence of the heterodimer. XPC-HR23B binds with high specificity to DNA-damage (7), nonetheless, the complex does not confer specificity to the TFIIH-associated ATPase for damaged-DNA, which may be due to the strong binding capacity of the XPC-complex to undamaged double-stranded DNA as well (30). The observation that XPA has affinity for TFIIH (31-33) is extended by our findings that XPA strongly stimulates the TFIIH-associated ATPase activity in the presence of DNA damage. This may suggest that the interaction of XPA and non-damaged DNA is simply not stable enough or is not able to induce the proper protein-DNA conformation to further activate the ATPase of TFIIH. The observation that XPA and XPC-HR23B stimulate the ATPase activity of TFIIH is in agreement with the notion that both proteins are actually involved in the unwinding step either sequentially or simultaneously.

Reversible serine/threonine phosphorylation of NER factors is implicated with efficient dual incision (27). In this report, a novel potential target for phosphorylation is identified, XPG, which is a substrate of the TFIIH kinase at least *in vitro*. Presently, it is not clear whether or not the TFIIH kinase activity is required for NER. Microinjection of anti-CDK7 antibodies inhibit NER *in vivo*, suggesting that CDK7 is involved in repair (19). However, it was also reported that TFIIH with or without the kinase catalytic subunit CDK7 is equally active in highly defined dual incision reactions *in vitro* (6). If the TFIIH-associated kinase proves indeed dispensible for NER in the dual incision step, phosphorylation may be important during post-incision events during, for instance, recycling or release of NER factors such as XPG from reaction intermediates.

Surprisingly, TFIIH inhibits the endonuclease activity of both XPG and ERCC1-XPF. Based on the reaction stoichiometry, the specific inhibition is likely mediated via ternary complex formation on DNA. Addition of ATP resulted in relief of repression of nuclease activity, which may involve -in case of XPG- phosphorylation. It is unlikely that ATP induced significant TFIIH-mediated unwinding because the incision sites of the model substrates were not clearly altered, and the availability of a restriction enzyme site was not affected by the presence of TFIIH and ATP. A possible mechanism to explain the observed effect is that ATP induces a conformational change in the nuclease-TFIIH-DNA ternary complex, involving displacement of TFIIH from the single-strand/double-strand junctions thereby relieving inhibition of nuclease activity. A role for TFIIH and ATP in reversible inhibition of ERCC1-XPF provides an attractive explanation for the defect in XP-B extracts derived from XP11BE cells containing TFIIH with a XPB protein mutated in the C-terminus. In these cell extracts, full opening around a DNA lesion is observed, but the formation of the 5' incision is defective (9), which may suggest that the carboxy-terminus of XPB is important for the ATP-dependent relief of ERCC1-XPF inhibition by TFIIH. To corroborate the involvement of the helicase subunits in the relief of repression, it will be of interest to test the effect of the isoquinoline derivative H-8, which specifically inhibits the kinase activity of TFIIH without affecting the DNA-dependent ATPase/helicase activities.

Together, these findings reveal additional roles for TFIIH and ATP, which have important implications for the mechanism of dual incision, and may involve phosphorylation of NER factors and conformational changes mediated by the TFIIH-associated ATPase activity distinct from DNA unwinding.

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CHAPTER 5

A Human Homolog of the Yeast Nucleotide Excision Repair Gene *MMS19*

Submitted for publication

A HUMAN HOMOLOG OF THE YEAST NUCLEOTIDE EXCISION REPAIR GENE *MMS19*

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Nucleotide excision repair (NER) is a DNA repair mechanism essential for the removal of UV-induced photoproducts, and a variety of chemical base-adducts and intra-strand crosslinks. The basic NER machinery is highly conserved from *Saccharomyces cerevisiae* to higher eukaryotes. *S. cerevisiae* Mms19 protein has a dual function in NER and RNA polymerase II transcription. Here we report the cloning and characterization of a putative human homolog. The human gene, tentatively designated *hMMS19*, encodes a 1030 amino acid protein with a predicted molecular mass of 113 kDa. The hMMS19 protein shares over its entire length 26 % identity and 51 % similarity to *S.cerevisiae* Mms19p and has virtually the same size. We identified structurally related polypeptides in mouse, rat, *Drosophila* and *C.elegans*. The degree of similarity between the mammalian MMS19 proteins and those of other organisms is consistent with the evolutionary distance between these species, indicating that the proteins are most likely true counterparts. The mouse *MMS19* gene is expressed as a single mRNA of 4.0 kb in a variety of mouse tissues with highest expression in testis. The human gene is localized on chromosome 10q24-10q25, a region that has not been involved in any disease implicated with UV sensitivity. As expected for a repair/transcription protein hMMS19 resides in the nucleus. Although the *hMMS19* cDNA failed to shown detectable complementation of the yeast *mms19Δ* phenotype, the data presented here indicate that also the MMS19 component of NER is strongly conserved throughout eukaryotic evolution.

INTRODUCTION

In spite of the chemical stability of DNA as a carrier of genetic information, the constant exposure of cells to numerous physical and chemical damaging agents leads to the formation of a wide diversity of DNA lesions. During evolution several DNA repair pathways have emerged in order to maintain the integrity of genetic information. The nucleotide excision repair (NER) pathway is one of the major processes to remove a broad range of structurally unrelated DNA lesions like UV-induced photoproducts, bulky chemical adducts and intra-strand cross-links (1). During eukaryotic evolution the basic 'cut and paste' mechanism of NER has been strongly conserved with obvious parallels between human and yeast and to a lesser degree even to prokaryotes. Thus *E. coli* uses a set of 6 proteins (UvrA-D, DNA polymerase I and DNA ligase) to eliminate NER lesions (2,3) while in eukaryotic cells 30 or so proteins are involved in the NER pathway (4-6). Genome-wide lesion detection is carried out by the XPC-hHR23B complex (7), presumably assisted by

the UV-DDB (XPE) dimer for the recognition of UV-induced cyclobutane pyrimidin dimers (CPD) (8). Lesions in the transcribed strand of active genes, that block ongoing transcription, are likely detected by an elongating RNA polymeraseII complex (9). Subsequently, the multi-subunit TFIIH factor, the damage verifier XPA and the single-strand DNA binding protein trimer RPA are recruited to the site of the lesion (6,10-12). The bidirectional helicase activity of TFIIH facilitates the formation of a locally opened repair intermediate (13-16). Then a dual incision is made around the lesion by structure-specific endonucleases XPG responsible for the 3' incision (17) and the ERCC1-XPF complex making the 5' cut (18). This allows excision of a 24-32mer oligonucleotide containing the damage (19). The last step of gap-filling is accomplished by general replication factors PCNA, RPA, RPC, DNA polymerase and DNA ligase sealing the final nick. The NER process acts on the entire genome, nevertheless there is a heterogeneity in the efficiency with which DNA damages are excised. It has been noted that lesions (such as CPD's) for which the global genome NER is slow are removed with a higher efficiency when they are present in the transcribed strand of active genes (20,21). Since the non-transcribed strand is repaired with an efficiency comparable to that of the global genome, this points to a direct link between the transcription apparatus and repair. Cells carrying mutations in the *CSA* and *CSB* genes suffer from a selective defect in this transcription-coupled NER (TC-NER) subpathway (22-24). Therefore, attention has focussed on CSA and CSB as TC-NER factors. CSA interacts with TFIIH (22) whereas CSB interacts with the RNA polymerase II complex (25), XPA (26) and XPG (27).

Mutations in the genes coding for NER factors cause genetic disorders such as xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (28,29). XP, a rare autosomal recessive condition, is mainly characterized by a dramatically high sun(UV)-sensitivity in addition to a strong predisposition to the development of skin cancer as well as in many patients accelerated neuro-degeneration. The CS and TTD patients are not particularly cancer-prone but present sun sensitivity, pronounced developmental abnormalities and very severe neurological problems due to neurodysmyelination. The additional hallmarks of TTD include ichtyosis as well as brittle hair and nails. The heterogeneous spectrum of clinical manifestations of CS and TTD can not be entirely accounted for by deficiencies in the NER process. Since all genes implicated in TTD encode subunits of the dual functional repair-transcription factor TFIIH, it has been postulated that part of the clinical features are the consequence of a subtle defect in basal transcription (30,31).

Although the complexity of the NER process is slightly higher in human than in yeast, the primary sequence of the proteins involved in DNA repair is largely conserved between both species, each yeast gene having, in general, at least one human counterpart. A mutation in one of the NER genes leads in most cases to the same molecular defect in both species. In recent years functions in the reaction mechanism have been assigned to all core NER factors. However, an additional

category of UV-sensitive yeast mutants exists disclosing genes which also appear *in vivo* to be involved in NER, but are to some extent dispensable for the NER reaction *in vitro*. This class is comprised of *RAD7*, *RAD16*, *RAD23* (32) and *MMS19* (33) and is functionally much less well understood. With the exception of Rad23p (34) no human homologues are known suggesting that they may be less well conserved during eukaryotic evolution. Here we focused on the *MMS19* gene. The *mms19Δ* mutant exhibits a moderate UV-sentivity due to a NER defect (33,35). The two subpathways of NER, global genome repair and TC-NER, are defective in a *mms19Δ* mutant (35). The pleiotropic phenotype of the mutant also involves a thermosensitive transcriptional activity as well as a methionine auxothrophy. A five minutes treatment of a total yeast extract at the non-permissive temperature leads to a strong reduction of RNA polymerase II transcription activity. Remarkably, the transcription defect can be corrected by highly purified TFIIH (19). As mentioned this 9-subunit complex, including the XPB and XPD helicases, is implicated in basal transcription initiation and is also required for NER (14,15,31,36). Mms19p may promote the function of TFIIH by allowing it to switch between both processes or by determining its stability. Thus, Mms19p may not be directly involved in these two processes but may influence them via TFIIH as an upstream regulatory factor. Here we report the identification and partial characterization of a presumed human homolog of Mms19p.

MATERIALS AND METHODS

Isolation of a cDNA encoding a putative human homolog of yeast MMS19

A HeLa poly(A)$^+$ cDNA library constructed in λgt10 with relatively long inserts (34) was screened with a probe described below. Approximately 600,000 recombinant bacteriophage plaques were transferred to Hybond-N membranes in duplicate. The probe used to screen the library was obtained by RT-PCR using random synthetic oligonucleotides for cDNA synthesis from HeLa total RNA. The oligonucleotide primer pairs used for amplification were: p274 5'-CATCCTGTTATTTCCCTATCG-3' and p276 5'-ACAAACACGCAGTCAGGGAG-3'. The 345 bp RT-PCR product was partially sequenced and confirmed to correspond to the amino acid sequence of EST c18436, purified from a 2% agarose gel, and labeled with [^{32}P]dATP and random hexamers. Hybridization was carried out in a solution of 10x Denhardts (1x: 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrolidone), 3x SSC (1x: 0.15 M NaCl, 15 mM Na-citrate), 0.1% SDS, 50 μg/ml salmon sperm DNA, 9.0% dextran sulphate overnight at 65°C. Membranes were washed for 20 minutes in 3x SSC/0.1% SDS, 1x SSC/0.1% SDS, and 0.3x SSC/0.1% SDS at 65°C and exposed to autoradiographic films with intensifying screens at -70°C.

RNA isolation and Northern blot analysis

Total RNA was isolated by the LiCl-urea method described (37). RNA samples were loaded onto a 0.9% agarose-formaldehyde gel, separated by electrophoresis and transferred onto a nitrocellulose membrane. The blot was probed with ^{32}P-labeled random-primed DNA corresponding to the open reading frame of the cDNA isolated above. The hybridization of the filter was performed at 42°C in 50% formamide, 5X SSPE, 1% SDS, 5X Denhardt's solution and 100µg/ml denatured salmon sperm DNA (38). The blot was washed at 65°C, two times 30 minutes in 1X SSC containing 0.1% SDS and exposed for autoradiography.

Subcellular localization

Adherent Cos-1 cells were transiently transfected using a mammalian expression vector designated pcDNA3-hMMS19. After incubation for 48 hours to allow expression of the cDNA insert, the cells were fixed with 4% paraformaldehyde. The encoded protein was localized by immunocytochemistry using a monoclonal antibody (1E11) raised against the N-terminal part of the encoded protein tentatively designated hMMS19. The second anti-mouse antibody was linked to the Hoechst fluorochrome which emits a red signal upon UV light irradiation. The nuclei were stained with DAPI.

Fluorescent in situ hybridization (FISH)

Human lymphocyte metaphase spreads were processed prior to hybridization as described (39). Briefly, the metaphase spreads were treated with 100µg/ml RNAse A in 2X SSC during 1h at 37°C, rinsed and incubated with proteinase K (20mg/100ml at 37°C) for 1h. The chromosomes were fixed with 1% formaldehyde, washed, dehydrated in ethanol and air-dried. The slides were hybridized in a mixture containing 50% formamide, 2X SSC, 40 mM Na-phosphate (pH 7.0), 10% dextran sulfate, 0.1 mg/ml sonicated salmon sperm DNA, 0.1 mg/ml yeast tRNA and 2 ng/ml labeled probe. The biotin-labeled complete ORF used as a probe was denatured at 80°C for 5 minutes and hybridized to the chromosomes overnight at 37°C. After a wash with 50% formamide in 2X SSC at 45°C followed by a second wash with 2X SSC containing 0.05% Tween 20 at room temperature, the slides were incubated with 5mg/ml avidin D-FITC (Vector, USA). To increase the sensitivity of the D-FITC signals, one round of amplification was performed using biotinylated goat anti-avidin D antibody. To generate clear reverse bands, metaphase chromosomes were either counterstained with propidium iodide in antifade medium or banded with DAPI and actinomycin D.

RESULTS

Identification, cloning, and sequence analysis of a human structural homolog of S. cerevisiae MMS19

The *S. cerevisiae* Mms19 protein sequence was used to systematically screen several public databases using the BLAST algorithm (40) to search for higher eukaryotic homologs of this protein. A computer translated partial cDNA clone derived from human placenta (Genbank accession number c18436) attracted our attention because it shared an intriguing level of homology with a portion of the *S. cerevisiae* Mms19 protein at amino acid positions 216 to 377. To see whether the homology extended, a RT-PCR-derived probe was used to screen a HeLa poly(A)$^+$ cDNA library. The nucleotide sequence of the longest cDNA clone isolated (3.4kb) showed that it was derived from the expected mRNA (see materials and methods for further details).

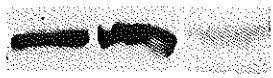
The sequence predicts a large ORF of 3090 base pairs encoding a protein of 1030 amino acids (Fig. 1A) with a calculated molecular weight of 113.07 kDa consistent with the observed electrophoretic mobility on SDS-PAGE of the *in vitro* translated protein (Fig. 1B). The assignment of the start codon is tentative since only limited information is available from the 5' sequence and no in frame stop codon is present in this region. However, several arguments are in favour of the idea that this is the correct start codon. First, the assumed start codon coincides well with the position of the start codon in *MMS19* (see below). Second, the encoded protein has a deduced size similar to that of the normal gene product in WCE of HeLa cells (Fig. 1B). Third, the *in vitro* translated and the recombinant protein purified from Baculo-infected cells co-migrate with the HeLa protein (Fig. 1B). Finally, we noticed the presence of a G at the positions +4 and -3, two critical nucleotides that make the recognition of the initiator codon more effective (41).

Although the cDNA was isolated from a poly(A)$^+$ library, neither a polyadenylation site was present nor a poly(A) tail. Nevertheless, a potential alternative polyadenylation site can be identified in the 3'-UTR (Fig. 1A).

The hMMS19 polypeptide contains 10.1% acidic (asp and glu), and 10.1% basic (arg, lys and his) amino acids and has a pI of 6.23. The charged residues are widely distributed throughout the protein. Furthermore, it is worth noting that the protein is rich in hydrophobic amino acids: 16.7% of the residues are leucines. Also striking is the presence of highly hydrophobic (leucine-rich) regions mainly in the N-terminal and C-terminal parts, containing 18 trimers of hydrophobic residues frequently preceded and/or followed by proline, glutamic acid or histidine residues (Fig. 1A). The encoded protein has a very alanine-rich N-terminus. Using a 3D structure prediction program amphiphatic helical wheel-like motifs were found. One side of this type of helix is mainly composed of polar residues while the opposite side contains essentially hydrophobic residues. These motifs are mostly

A

B



C

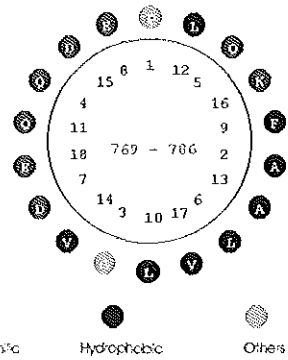


Figure 1. (A) Nucleotide and deduced amino acids sequence of *hMMS19*. The numbers to the left denote amino acid position (lower numbers) and the nucleotide position (upper numbers). The translation stop codon TGA at nucleotide +3091 is marked with an asterisk. Hydrophobic trimers frequently flanked by proline, glutamic acid or histidine residues are underlined. In bold is a potential alternative polyadenylation site. The potential nuclear localization signal is double underlined. Amino acids are indicated using the universal one-letter code. (B) Immunodetection of the endogenous and baculovirus recombinant hMMS19 protein (lanes 1 and 2) using anti-hMMS19 antibodies. Lane 3 shows the *in vitro* transcribed-translated hMMS19. Similar molecular weight is observed for each. (C) Identification of potential amphiphatic helical-wheel like motif in the hMMS19 amino acid sequence. The potential 3D motifs were obtained using the Antheprot V3_5b programme. Amino acids are plotted every 100° consecutive around the spiral. In dark grey is an amino acid with a polar side chain; in black is a hydrophobic side chain. The helix is polar on one side and hydrophobic on the other side.

concentrated in the last 360 C-terminal amino acids (between residues 337 and 355, 670-688, 769-787, 841-859, 890-908, 995-1013) (for an example see Fig. 1C). A putative nuclear localization signal, KKKXXRK, is present in the very C-terminal part of the ORF. No other known motifs were found using the Antheprot V3_5b program except for several potential casein kinase II and protein kinase C phosphorylation sites.

The ORF sequence was compared with that of the yeast *MMS19*. A striking correspondence in size was noted (1030 versus 1032 amino acids for the human and yeast products respectively). Furthermore, co-linear highly significant homology was found over the entire sequence. Overall the human protein is 26% identical and 51% similar to yeast Mms19p. The N-terminus (from residue 1 to 310) as well as the C-terminus (from residue 794 to 1030) are better conserved than the central part (311-793) with 28%, 33% and 22% of identity and 51%, 55% and 48% of similarity, respectively. When the complete human ORF was screened against the entire *S. cerevisiae* protein database using the BLAST algorithm Mms19p was identified as the by far most homologous protein ($p=2.10^{-41}$). This indicates that within the yeast genome Mms19 is the only counterpart. On the basis of the strong overall homology and on additional findings and arguments given above we henceforth designate the gene as "hMMS19". Lauder *et al.* (33) reported the presence of 15 leucine-rich motifs (XXLXXLXXLXXLXLXXNXLXXL) between the residues 588 and 880. Although the numbers of hydrophobic residues and especially leucine residues, for both species, are equivalent in the 588-880 region, the leucine-rich motifs as defined above present in the yeast protein are poorly conserved. Using the human hMMS19 sequence we screened several available electronic databases and identified fragments of genes in various other species with high amino acid sequence homology to the *hMMS19* ORF. These are compiled in Fig. 2. The alignment of the various homologous proteins allowed the construction of a phylogenetic tree. The degree of similarity between the different proteins matches well with the overall degree of evolutionary relatedness of the species involved. These findings lead us to conclude that in all species there is one clear homolog to ScMMS19.

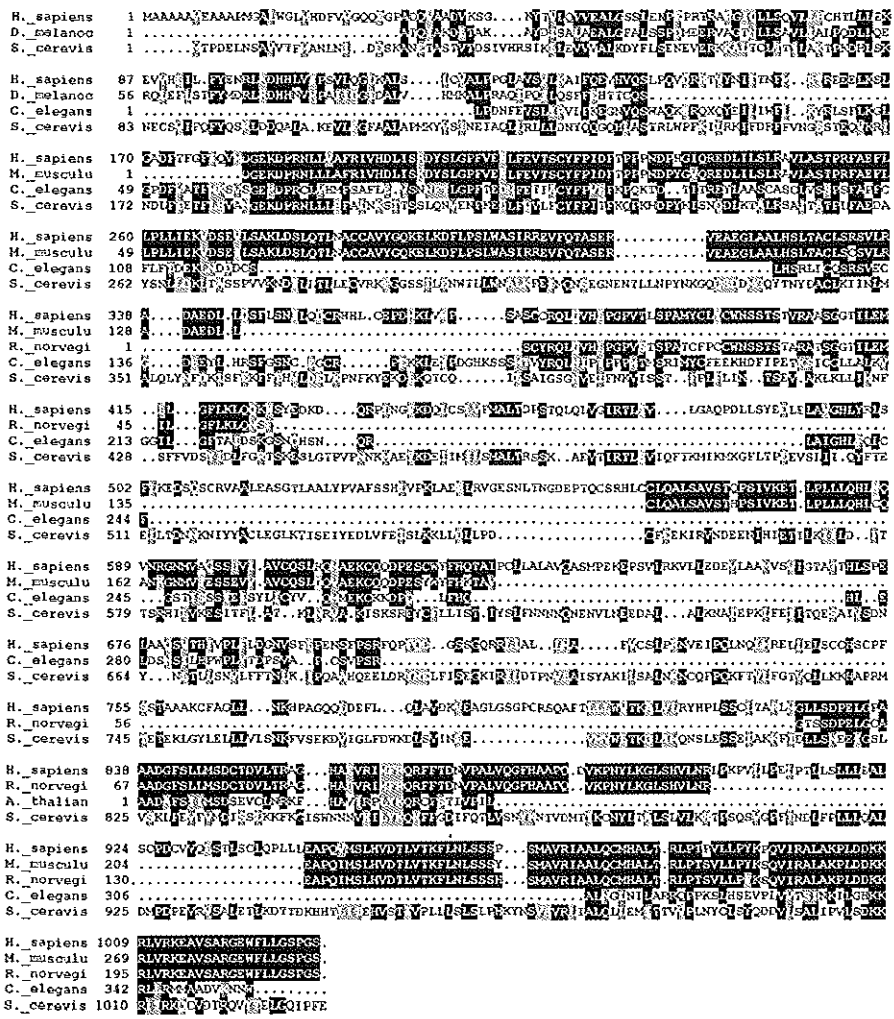


Figure 2. Comparison of amino acid sequences of the human hMMS19 with its yeast counterpart and homologous translated ESTs of different species: *Homo sapiens* (Hs), *Mus musculus* (Mm), *Rattus norvegicus* (Rn), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), *Arabidopsis thaliana* (At) and *Saccharomyces cerevisiae* (Sc). Highly conserved amino acids are boxed in black. Related residues are shaded in grey.

Expression pattern of hMMS19

To estimate the transcript size as well as its expression pattern, Northern blot analysis was carried out using total RNA from a variety of mouse tissues and HeLa

cells. Fig. 3 shows that the cDNA hybridized to a single transcript of 4 kb in the various mouse tissues and in human cells. The β-actin hybridization as well as the ethidium bromide staining of the RNA reflect the relative amounts of total RNA loaded into each slot. We conclude that the gene is expressed in all tested organs and tissues at a rather low level except in testis where the mRNA appears to be much more abundant. Furthermore, various ESTs from databases confirmed that this gene is widely expressed in human and mouse tissues. *hMMS19* mRNA is found in skin, placenta, cerebellum, mammary gland, parathyroid gland, myotubes, uterus and at all stages of development (blastocyst, embryo, fetus as well as adult tissues).

Since only one band was observed in mRNA samples, we conclude that there is no alternative splicing of major parts of the *hMMS19* gene. Obviously alternative splicing of small exons can not be detected by this type of analysis.

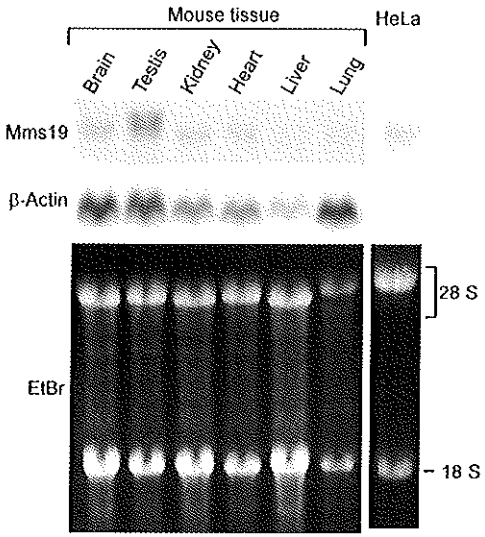


Figure 3. Northern blot analysis of *hMMS19*. Hybridization was achieved with a specific *hMMS19* or b-actin (as an internal control) cDNA probe, as described in materials and methods. The lower part shows the amount of total RNA loaded on the agarose gel. A single transcript of 4 kb is observed.

Chromosomal localization

To further characterize *hMMS19* and its potential involvement in human disorders, chromosomal localization was carried out. The cDNA encompassing the complete ORF was biotinylated and used as a probe for *in situ* hybridization to human metaphase spreads. A single site of hybridization on the human karyotype was found located on the long arm of chromosome 10 at the junction of q24 and q25 (Fig. 4).

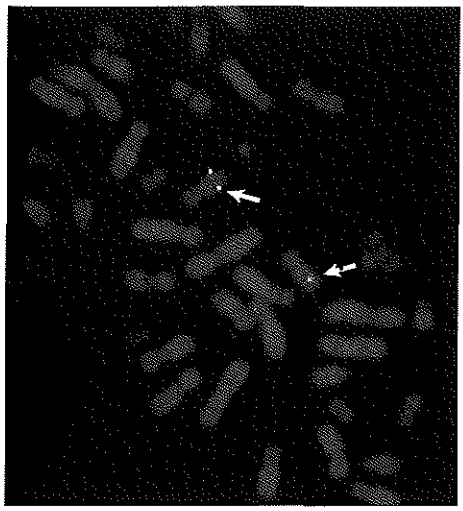


Figure 4. Chromosomal localization of hMMS19 by fluorescent *in situ* hybridization. The biotinylated *hMMS19* cDNA was hybridized with a spread of human metaphase chromosomes that were thereafter counterstained with propidium iodide. The arrows indicate the fluoresecent spots present in the region 10q24-10q25 of the R-banding pattern.

Subcellular localization

The subcellular localization of hMMS19 protein was investigated by transfecting Cos cells with a pcDNA3-hMMS19 construct. An anti-hMMS19 monoclonal antibody raised against a N-terminal peptide, was used to reveal the presence of the protein. Fig. 5 shows a clear nuclear localization of the protein in transfected cells only. A second antibody raised against the C-terminus confirmed the same exclusively nuclear localization of hMMS19 (data not shown).

Interspecies complementation

In order to know whether the human protein is able to complement the yeast defect, a *mms19Δ* strain (MGSC217) in which almost the complete ORF of *MMS19* is replaced by the *URA3* gene, was transformed with the hMMS19 cDNA subcloned in the yeast expression vector, pYET2 (35). UV-sensitivity, thermosensitivity as well as methionine auxotrophy of this transformed strain were examined. We failed to observe any significant rescue of the cells for any of the defects, although the human protein could be detected as a weak band in the transformed yeast extract by immunoblot analysis using monoclonal antibodies raised against the human protein (data not shown). These findings suggest that the human protein has diverged too

much from its yeast counterpart to be able to complement the various *mms19Δ* deficiencies.

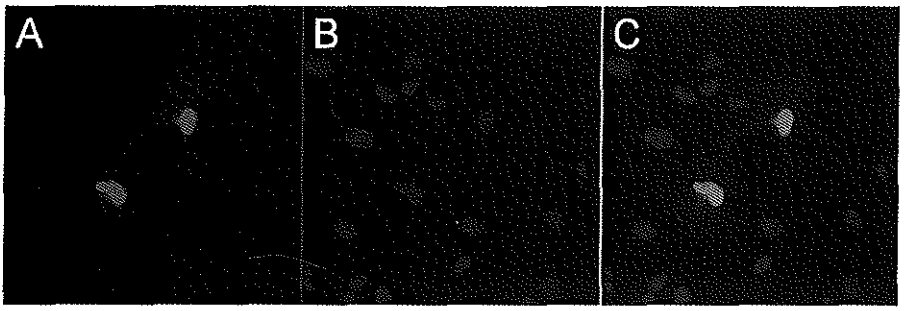


Figure 5. Subcellular localization of the hMMS19 protein. Wild type Chinese hamster ovary cells were transfected with a pcDNA3-hMMS19 construct. The hMMS19 protein was detected by immunocytochemistry using anti-hMMS19 antibodies. The central picture shows the DAPI staining of the nuclei, the left one reveals the localization of hMMS19 and the right panel shows a combination of both stains. The protein is exclusively present in the nucleus.

DISCUSSION

The analysis of the phenotype of a collection of UV-sensitive *S. cerevisiae* mutants revealed the existence of a significant number of genes involved in nucleotide excision repair. These mutants belong to the RAD3 epistasis group. Studies on three human genetic disorders, xeroderma pigmentosum, Cockayne syndrome, trichothiodystrophy and a series of UV-sensitive Chinese hamster mutants, highlighted the structural and functional conservation of the NER genes from yeast to mammals. Although the mammalian NER process is more complex than that of the yeast, the main features are strikingly similar. Thus, several human NER genes were isolated on the basis of homology with their yeast counterpart and *vice versa* (18,34,42-44).

Using a human EST potentially homologous to yeast Mms19p to screen a HeLa library, we have isolated a unique human cDNA. The deduced protein sequence and Mms19p show an overall co-linear homology and a remarkable similarity in size (113.07 kDa in human and 117.91 kDa in yeast). Moreover *MMS19* is the only gene in the yeast genome that exhibits such a highly significant homology when the ORF is used to screen the entire yeast genome database. The level of sequence similarity and identity is in the same range as found for a number of other NER genes. Furthermore, it is worth noting that the degree of homology found between the homologous proteins of different organisms matches the evolutionary distance of the various species. The ubiquitous expression of the protein as well as its nuclear localization are entirely consistent with its proposed function in DNA repair and

possibly in transcription. Taken together these findings support the idea that the isolated cDNA corresponds to the human homologue of the yeast *MMS19* gene. However, it is not excluded that other MMS19 homologs exist in the human genome as is the case for some other repair/transcription genes such as *RAD6* (45), *RAD23* (34) and *SSL1* (46).

The human gene failed to complement the yeast mutant in spite of the fact that we could detect expression of the human protein. It is not excluded that the very low level of expression of the human protein was insufficient to rescue the cell. This is consistent with the notion that only a few human NER genes have been reported to correct (part of) the corresponding yeast mutant phenotype (complementation of *rad3* mutant yeast by the human *XPD* gene (47), complementation of the *S.pombe swi10* mutant yeast by the human *ERCC1* gene (48)).

Although the human protein contains very hydrophobic regions, the leucine-rich motifs identified in yeast Mms19p are not conserved in the human polypeptide, indicating that these regions may not be essential for *MMS19* function. Both extremities especially the C-terminus appear better conserved. Also, different amphiphatic helical wheel-like motifs are present in both proteins but not always at the same locations. Such motifs could be involved in protein-protein interactions possibly via hydrophobic interactions.

The gene is located in a region of chromosome 10q24-25 to which a relatively high number of genes have been assigned. One of these is hTAFII100 which encodes a subunit of the RNA polymerase II transcription initiation factor TFIID (49). Two genes encoding two proteins involved in DNA repair reside in the vicinity of the *hMMS19* locus, *ERCC6* (or CSB) (50) and O(6)-methylguanine-DNA methyltransferase (51,52). The first is required for accurate coupling of the RNA polymerase II transcription to NER while the second removes the O(6)-alkylguanine in DNA which is the major mutagenic and carcinogenic lesion in DNA induced by simple alkylating mutagens due to its preference for pairing with thymine during DNA replication.

Sutherland (53) and Scheres et al. (54) identified a new class of fragile sites at 10q25 that requires bromodeoxyuridine for expression. This fragile site which appears to be inherited in a Mendelian dominant manner is present in approximately one in 30 of the Australian population and has only been observed in heterozygotes. However, no genetic disorders related to UV-sensitivity were attributed to genes situated in the proximity of the *hMMS19* locus.

Little is known about the role of MMS19 in transcription and in NER. TFIIH of a wild type yeast strain can correct the transcriptional defect of a yeast *mms19Δ* extract which is -in addition- NER-defective. The cloning and expression of human *MMS19* will enable a combined genetic and biochemical approach to further elucidate the function of this gene and its interactions with other components of RNA polymerase II transcription and NER machinery.

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SUMMARY AND CONCLUSIONS

Although DNA appears as an extremely stable molecule at the macromolecular level, it is subject to continuous changes. DNA lesions are introduced both under endogenous cellular conditions, as well as by environmental factors, such as chemicals that are reactive with base groups, or physical agents like UV or ionizing radiation. These damages can directly interfere with DNA metabolism and result in abnormal cell behavior, cell death, or induction of permanent alterations of the genetic material.

DNA repair processes prevent the detrimental effects of DNA lesions. Nucleotide excision repair (NER) is one of the major DNA repair pathways essential for the removal of UV-induced photoproducts and numerous bulky adducts from DNA. The basic mechanism is conserved from prokaryotic to human cells and can be divided into two major stages: recognition and dual incision of the damaged strand on both sides of the DNA lesion, and subsequent gap-filling repair DNA synthesis by the replication machinery. Six human protein factors are required for recognition and dual incision *in vitro*. The XPC-HR23B complex binds with high specificity to DNA damage and represents the initial recognition factor able to recruit the remainder of the NER apparatus. Three additional factors are required for the subsequent ATP-dependent local melting of the DNA helix around the lesion: basal transcription factor IIH (TFIIH), which has an additional, essential cellular function in transcription by RNA polymerase II; XPA, which preferentially binds to DNA containing lesions as well as single-stranded DNA; and the single-stranded DNA binding protein RPA, which most likely binds to the undamaged strand. The locally unwound region provides a substrate for the structure-specific endonucleases XPG and ERCC1-XPF, which incise the damaged strand at the 3' and 5' side, respectively.

In **chapter 1**, the mechanism of NER and relevant aspects of transcription by RNA polymerase II are reviewed. Strong emphasis is put on the pivotal role played by TFIIH in both processes and biochemical aspects involved. The principal findings described in the experimental part of this thesis are also integrated and discussed in this chapter. Nine subunits constitute TFIIH, including the DNA helicases XPB and XPD. Mutations in these subunits of TFIIH are associated with three inheritable syndromes: xeroderma pigmentosum, a combined form of xeroderma pigmentosum and Cockayne syndrome, and a photosensitive variant of trichothiodystrophy. In the subsequent **chapters 2-5**, experimental work is described that addresses the function of TFIIH in both transcription and DNA repair. **Chapter 2** describes the development of a novel purification procedure for TFIIH facilitating the biochemical characterization of this complex. A cell line stably expressing tagged XPB was generated allowing the immunopurification of the XPB protein and associated factors. Addition of two tags, a N-terminal hexameric histidine stretch and a C-terminal HA epitope, to this highly conserved

protein did not interfere with its function in DNA repair and transcription. The HA epitope allowed efficient TFIIH purification using a relatively simple two-step immuno-affinity procedure. The purified complex exhibited all activities associated with TFIIH: DNA-dependent ATPase, helicase, CTD kinase, and participation in *in vitro* and *in vivo* NER and *in vitro* transcription. From this analysis, we conclude that the predominant active form of TFIIH is composed of nine subunits both in DNA repair and transcription and that there is one molecule of XPB per TFIIH complex.

By modifying this procedure, we purified TFIIH carrying a wild type or an active-site mutant epitope-tagged XPD subunit (**chapter 3**). In contrast to XPB, XPD helicase activity was largely dispensable for *in vitro* transcription, formation of short (tri-nucleotide) transcripts and promoter opening. Moreover, microinjection of mutant XPD cDNA did not interfere with *in vivo* transcription. These data showed directly that XPD activity is not required for transcription. However, with respect to DNA repair, neither 5' nor 3' incisions in defined positions around a DNA adduct were detected in the presence of TFIIH containing inactive XPD, although substantial damage-dependent DNA synthesis is induced in the presence of mutant XPD both *in vitro* and *in vivo*. This indicates that mutant XPD causes aberrant damage-dependent DNA synthesis without effective repair, consistent with the discrepancy between repair synthesis and survival after DNA damage in a number of cells from XP-D patients. Thus, there is a differential requirement for the enzymatic activity of the XPD helicase subunit in NER and RNA polymerase II transcription.

Further analysis of the function of TFIIH in NER is presented in **chapter 4**. As TFIIH is implicated in the repair reaction with local DNA unwinding attributed to its two helicase subunits, XPB and XPD, the complex may have additional roles during the repair reaction. To further define the role of TFIIH in NER, functional interactions between TFIIH and other DNA repair proteins were analyzed. We show that the ATPase activity by the TFIIH-associated helicase subunits is stimulated both by XPA and the XPC-HR23B complex. However, while XPA promotes the ATPase activity specifically in the presence of damaged-DNA, stimulation by XPC-HR23B is dependent on DNA without additional specificity for lesions. Secondly, we demonstrate that the TFIIH-associated kinase activity is able to phosphorylate XPG *in vitro*. Finally, using model substrates that mimic NER intermediates, we reveal that TFIIH inhibits the structure-specific endonuclease activities of both XPG and ERCC1-XPF, responsible for the 3' and 5' incision in NER, respectively. The inhibition occurs in the absence of ATP, and is reversed upon addition of ATP. The ATP-reversed inhibition may in part be mediated via the DNA-dependent ATPase activity associated with the XPB and XPD subunits and point towards additional roles for TFIIH and ATP in NER distinct from a requirement for DNA unwinding in regulating the endonucleolytic activities of XPG and ERCC1-XPF. In the final **chapter 5**, the cloning of the cDNA encoding a human structural homolog of yeast *MMS19* is reported. The *S. cerevisiae* Mms19 protein has a dual function in NER

and RNA polymerase II transcription as a regulatory factor. The human *MMS19* (*hMMS19*) gene encodes a nuclear protein with a predicted molecular mass of 113.07 kDa. The hMms19 protein is 51% similar (26% identical) to its yeast counterpart. The gene is expressed as a single mRNA of 4.0 kb in a variety of mouse tissues with highest expression in testis and localized on chromosome 10q24-10q25, a region that has not been involved in any disease related to UV-sensitivity. Although the hMMS19 cDNA failed to complement the yeast *mms19Δ* phenotype, the data presented indicate that not only the basic NER proteins, but also regulatory factors of this repair machinery are conserved between yeast and mammalian cells.

SAMENVATTING

DNA is de drager van het erfelijk materiaal. Het DNA molecuul bestaat uit twee in elkaar gevlochten strengen, die zorgvuldig worden gekopieerd en overgedragen van cel tot cel gedurende vele generaties. De strengen zijn opgebouwd uit vier bouwstenen, die aangeduid worden met de letters A, T, G en C. Als een streng op een bepaalde positie bouwsteen A bevat, bezit de tegenoverliggende streng altijd bouwsteen T en omgekeerd. Ditzelfde geldt voor de bouwstenen G en C. Op deze manier is een DNA streng altijd een kopie van de tegenoverliggende (complementaire) streng. Het DNA in menselijke cellen bevindt zich in de celkern en bevat circa 100.000 genen, die van elkaar verschillen doordat de volgorde van de bouwstenen A, T, G en C in elk gen anders is. Als een gen actief is in een cel, wordt het overgeschreven (dit wordt transcriptie genoemd). Het overgeschreven gen (boodschapper-RNA) wordt vervolgens vervoerd naar het omliggende cytoplasma van de cel waar het vertaald wordt in een eiwit. In cellen zijn niet alle genen tegelijkertijd actief, maar slechts een bepaalde set kenmerkend voor het celtype.

Ondanks het feit dat het DNA een zeer belangrijk molecuul is, wordt het voortdurend beschadigd. Deze beschadigingen vinden plaats onder normale omstandigheden in een cel, bijvoorbeeld door de aanwezigheid van zuurstof, of worden veroorzaakt door externe factoren, zoals chemicaliën die reageren met DNA of straling afkomstig van zonlicht of Röntgen bronnen. DNA schade kan leiden tot afwijkend gedrag van een cel, celdood, of een permanente verandering in het DNA (mutatie). Een mutatie kan in bepaalde gevallen de oorzaak zijn van kanker of van een erfelijke ziekte, als de mutatie plaatsvindt in een geslachtscel.

Diverse DNA herstel mechanismen voorkomen de nadelige effecten van DNA schade. Het nucleotide excisie reparatie (NER) mechanisme is een van de belangrijke DNA herstel processen. Het herkent en verwijdert vele typen schade, die veroorzaakt worden door bijvoorbeeld sigarettenrook, uitlaatgassen en zonlicht (UV). Het mechanisme komt voor in verschillende soorten cellen, variërend van bacteriën en bakkersgist, tot menselijke cellen. Het belang van het NER proces is duidelijk zichtbaar bij patiënten die een defect hebben in dit DNA herstel mechanisme. Zo hebben patiënten die lijden aan de erfelijke ziekte xeroderma pigmentosum (XP) een extreme overgevoeligheid voor zonlicht, een droge, leerachtige huid, pigmentatie afwijkingen, en een zeer sterk verhoogde kans op het krijgen van huidtumoren. Andere zeldzaam voorkomende syndromen die gekenmerkt worden door gevoeligheid voor zonlicht zijn Cockayne syndroom (CS) en een fotogevoelige vorm van trichothiodystrofie (TTD). Deze syndromen worden gekenmerkt door groei en neurologische afwijkingen, en -in het geval van TTD- breekbare haren en nagels.

NER werkt als volgt: na herkenning van een schade in een DNA streng, wordt aan beide zijden van de beschadiging de aangedane DNA streng doorgeknipt en de laesie verwijderd. Vervolgens wordt het 'gat' opgevuld waarbij de

tegenoverliggende streng als voorbeeld dient. In menselijke cellen zijn voor de herkenning en verwijdering van een schade minimaal zes verschillende eiwitfactoren nodig: XPA, RPA, XPC-HR23B, TFIIH, en de eiwitten XPG en ERCC1-XPF, die de eigenschap bezitten dat ze een DNA streng kunnen doorknippen.

In dit proefschrift staat de bestudering van TFIIH centraal. TFIIH is een eiwitfactor, dat is opgebouwd uit negen verschillende subeenheden. Een bijzondere eigenschap van TFIIH is dat deze eiwit-factor niet alleen nodig is voor NER, maar ook een essentiële functie heeft in transcriptie, het overschrijven van genen. De twee grootste subeenheden van TFIIH, XPB en XPD, hebben de eigenschap dat zij DNA strengen kunnen ontwinden. Hiervoor hebben XPB en XPD het ATP molecuul nodig, dat de beide subeenheden voorziet van energie. Het ontwinden van DNA is een belangrijke stap in zowel het transcriptie als NER.

In **hoofdstuk 1** wordt de huidige literatuur betreffende de functie van TFIIH in NER en transcriptie beschreven. De daarop volgende **hoofdstukken 2-5** bevatten een beschrijving van het uitgevoerde onderzoek. Voor de klassieke zuivering van TFIIH zijn tenminste vijf opeenvolgende chromatografische scheidingen nodig waarbij zeer veel celmateriaal nodig is. **Hoofdstuk 2** beschrijft de ontwikkeling van een methode om TFIIH te zuiveren via twee relatief eenvoudige stappen uit een beperkte hoeveelheid gekweekte menselijke cellen. Hierdoor is het mogelijk om de samenstelling en de werking van het TFIIH complex gedetailleerd te bestuderen. Uit dit onderzoek is ook gebleken dat per TFIIH factor slechts één molecuul XPB aanwezig is. In **hoofdstuk 3** wordt deze methode toepast om mutant TFIIH te zuiveren. Deze vorm van TFIIH bevat een mutant XPD subeenheid, dat niet in staat is om DNA te ontwinden. Uit de karakterisering van dit mutant TFIIH blijkt dat DNA ontwinding door XPD niet nodig is voor transcriptie. Maar deze activiteit van XPD is wel noodzakelijk voor NER: in de afwezigheid van actief XPD wordt de beschadigde streng aan geen van beide zijden van een schade doorgeknipt. In **hoofdstuk 4** wordt de interactie tussen TFIIH en de overige vijf NER factoren onderzocht. Twee factoren -XPA en XPC-HR23B- stimuleren de benutting van het ATP energiemolecuul door TFIIH. In het geval van XPA is deze stimulatie bovendien afhankelijk van de aanwezigheid van beschadigd DNA. Een tweede opvallende bevinding is dat TFIIH de knipactiviteit van XPG en ERCC1-XPF tegengaat onder bepaalde omstandigheden. Deze inhibitie door TFIIH van de knipeiwitten kan worden opgeheven door toevoeging van het ATP energiemolecuul. Dit wijst op een nieuwe functie voor TFIIH tijdens de NER reactie. In het laatste **hoofdstuk 5** wordt de identificatie en de isolatie beschreven van een nieuw menselijk gen. Dit gen bevat de code voor een eiwit dat sterk overeenkomt met een eiwit uit bakkersgist, MMS19. In gistcellen kan het MMS19 eiwit de functie van TFIIH beïnvloeden. De isolatie van het humane gen, en de lokalisatie op chromosoom 10 maakt het mogelijk om eventuele patiënten met mutaties in dit gen op te sporen.

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NAWOORD

De laatste pagina van een proefschrift is voor velen de belangrijkste, of zelfs enige, pagina die met aandacht gelezen wordt. En niet ten onrechte, want een proefschrift is het resultaat van vele kleine en grote bijdragen van collega's, vrienden en familie. Het staat me nog goed bij hoe ik in Rotterdam terecht ben gekomen via Backey, mijn stage-begeleider in Leiden. Voordat ik het me realiseerde, bevond ik me in een sollicitatie gesprek met Geert Weeda en mijn promotoren Jan Hoeijmakers en Dirk Bootsma. Ik wil alle drie op deze plaats graag bedanken voor hun bijdragen aan mijn promotie-onderzoek. Vervolgens de lab-genoten van het eerste uur: Jan (pJdB, goede naam voor een plasmide), net drie maanden eerder begonnen aan een project dat ook met TFIIH te maken had, maar dan iets met muizen, Bert (van der worst, of ook wel GTJ Hiltermann) die met name in de eerste paar jaar elke maandag nieuwe moppen vertelde, en Henk, mede-forens naar Leiden, en ook tegenspeler in de derby VV Leiden 9 - Leidse Boys 1 (Uitslagen: 0-2, 3-3, 3-1 en 6-2). Ook de lab-genoten voor kortere tijd, Coen, Ingrid, Jose, Remco, Deb, en Suzanne hebben bijgedragen aan de gezelligheid in het lab. Tenslotte, nog drie collega's die een speciale vermelding verdienen. Als eerste Thierry, onze Fransoos. I can vividly remember the way you walked through the corridors of the IGBMC with a 'sausage' of dialysis tubing filled with extract derived from 100 litres of cells. I hope that you are -at least a little bit- proud of your contribution to this thesis. I enjoyed working with you and look forward to reading your thesis soon. De 'prutsers' Manja en Betty heb ik pas echt leren kennen na hun verhuizing naar 730. De spontaneïteit waarmee jullie de afscheidsborrel vlak voor mijn vertrek naar Engeland hebben georganiseerd, was hartverwarmend. Ik ben heel blij met jullie hulp als paranimfen. En Betty, ik hoop dat je na deze korte cursus goed voorbereid begint aan je eigen promotie. Overigens, ik heb nog geen vervanging gevonden voor jullie rol als koffie- resp. lunchgezelschap.

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Bas