Mutations in XPB and XPD helicases found in xeroderma pigmentosum patients impair the transcription function of TFIIH

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As part of TFIIH, XPB and XPD helicases have been shown to play a role in nucleotide excision repair (NER). Mutations in these subunits are associated with three genetic disorders: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). The strong heterogeneous clinical features observed in these patients cannot be explained by defects in NER alone. We decided to look at the transcriptional activity of TFIIH from cell lines of XP individuals. We set up an immunopurification procedure to isolate purified TFIIH from patient cell extracts. We demonstrated that mutations in two XP-B/CS patients decrease the transcriptional activity of the corresponding TFIIH by preventing promoter opening. The defect of XPB in transcription can be circumvented by artificial opening of the promoter. Western blot analysis and enzymatic assays indicate that XPD mutations affect the stoichiometric composition of TFIIH due to a weakness in the interaction between XPD–CAK complex and the core TFIIH, resulting in a partial reduction of transcription activity. This work, in addition to clarifying the role of the various TFIIH subunits, supports the current hypothesis that XP-B/D patients are more likely to suffer from transcription repair syndromes rather than DNA repair disorders alone.

Keywords: Cockayne syndrome/helicase/nucleotide excision repair/transcription factor IIH/xeroderma pigmentosum

Introduction

Until the discovery of a connection between TFIIH, a basal transcription factor, and rare genetic repair disorders, mutations in general transcription factors had not been thought to be responsible for such diseases (Friedberg, 1996; Hoeijmakers et al., 1996). TFIIH, which contains nine subunits, supplies several enzymatic activities in the two DNA helicases XPB and XPD, and in cdk7, the cyclin H-dependent kinase (Feaver et al., 1993; Schaeffer et al., 1993, 1994; Drapkin et al., 1994; Feaver et al., 1994; Guzder et al., 1994a,b; Roy et al., 1994a). XPB and XPD are thought to participate in DNA unwinding to allow either the gene to be transcribed by RNA polymerase II (RNA pol II) (Holstege et al., 1996) and/or the removal of DNA lesions induced by a large variety of genotoxic compounds including UV light and anti-cancer drugs (Evans et al., 1997).

It is, therefore, not surprising that mutations in TFIIH helicases were found to be associated with the human repair syndromes, xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). XP, a rare human hereditary syndrome, is characterized by hyperpigmentation of the skin under sun exposure, cutaneous abnormalities and predisposition to skin cancer, which are likely nucleotide excision repair (NER) phenotypes, and also clinical features such as developmental retardation, neurological abnormalities which cannot simply be explained by NER defects (Bootsma and Hoeijmakers, 1993; Vermeulen et al., 1994a). CS patients display mainly neurological and developmental abnormalities associated with sensitivity of the skin to sun, but have no predisposition to skin cancer. TTD is a non-cancer-prone disorder in which patients present abnormal facial features, mental retardation, brittle hair and DNA repair defects similar to XP.

One of the subunits of TFIIH, p44, is also found to be associated with human patient phenotypes unrelated to impairment of DNA repair. Indeed, one of the two p44 genes (p44t) is involved with the survival motor neuron gene (SMN) in large-scale deletions generating the most severe form of spinal muscular atrophy (SMA), also called Werdnig–Hoffman disease (Bürstlen et al., 1997). SMA is a frequently lethal autosomal recessive disorder characterized by degeneration of horn cells of the spinal cord and skeletal muscle denervation leading to paralysis.

The formation of an open DNA structure is TFIIH- and ATP-dependent in both transcription and NER. RNA synthesis from either a supercoiled DNA template (Parvin and Sharp, 1993; Goodrich and Tjian, 1994) or an artificially premelted promoter (Holstege et al., 1996; Dvir et al., 1997) could be performed in an in vitro reconstituted transcription system lacking TFIIH, whereas TFIIH is required when the template is linear. Some mutations in XPB and XPD can completely prevent opening and dual incision of the damaged oligonucleotide in NER (Evans et al., 1997). Moreover, XPB helicase activity appears to be more crucial than XPD helicase activity for transcription. In yeast, mutation in the ATP binding site of Rad25 (the homolog of XPB) results in defects in both transcription and NER (Park et al., 1992). In contrast, mutation in the ATP binding site of Rad3 (the homolog of XPD) does not affect viability of yeast but affects NER (Sung et al., 1988). TFIIH containing this XPD mutant is active in transcription (Feaver et al., 1993). These results suggest that the ATPase activity (and therefore the helicase activity) of XPD is dispensable for transcription but do not discount the possibility that other domains that function
in DNA binding or protein–protein interactions have an important role in transcription. In support, a temperature-sensitive mutation of Rad3 has been shown to lead to deficient transcription (Guzder et al., 1994a). In addition, it was demonstrated that XPD helicase activity was stimulated through its binding with p44 subunit (Coin et al., 1998). Mutations in the C-terminal domain (CTD) of XPD which prevent such interactions result in a decrease in the TFIIH helicase activity but could also modulate TFIIH composition and lead to transcription defects.

As all the clinical features of the patients cannot be explained on the basis of NER defects, we wondered if and how basal transcription is affected by the various TFIIH mutations. It was hypothesized that in addition to preventing interactions with transcriptional activators or mediators, these mutations might affect the helicase/ATPase as well as the kinase activities of TFIIH, and also the interactions with the other basal transcription factors, thus disturbing RNA synthesis. To understand the role of TFIIH in gene expression-related mechanisms, we aimed to dissect the effects of mutations in some of the TFIIH subunits which give rise to various clinical features and we particularly focused on the analysis of the consequences of these mutations in the transcription reaction. We first set up an easy and fast immunopurification procedure to isolate and characterize TFIIH in transformed cell lines from different patients in which XPB, XPD or p44 are mutated or deleted. We then tested the enzymatic activities of the various TFIIH complexes and analyzed the implication of XBP and XPD mutations in transcription and especially in promoter opening, using artificial premelted promoter and KMnO4 footprinting experiments. Together, our results point out the crucial role of XBP in transcription and sustain the hypothesis that XP-B and XP-D patients are more likely to suffer from transcription repair syndromes rather than DNA repair disorders.

Results

Immunopurification of TFIIH

In order to isolate TFIIH from patient cell lines, immunopurification was performed using monoclonal antibody raised towards p44 (Ab-p44), crosslinked to protein A–agarose (Figure 1A) (Humbert et al., 1994). To optimize our purification procedure, whole cell extracts (WCE) from HeLa cells were first fractionated on a heparin–Ultrogel column as described (Gerard et al., 1991). Ten milliliters of the 0.4 M KCl fraction were then incubated with the Ab-p44–protein A–agarose. The resin was extensively washed with the same buffer to eliminate the non-specific proteins and elution was performed overnight, using an excess of oligopeptide mimicking the first 17 amino acids of the N-terminal domain of p44 as a competitor. After dialysis, the eluted fraction [TFIIH(ip)] was analyzed by SDS–PAGE and Western blotting using antibodies generated towards each of the TFIIH subunits. Each polypeptide corresponds to the previously identified TFIIH subunits present in the HAP fraction (Marinoni et al., 1997; Figure 1B, SDS–PAGE and Western blotting, compare lane 1 with 2 and lane 3 with 4). The composition of both purified TFIIH subunits is quite similar. However, two polypeptides were present below the p62 subunit in TFIIH(ip), which are not yet microsequenced and are dispensable for TFIIH basal transcription activity. The lowest molecular weight polypeptide is insulin.

Using this immunopurification assay and starting with 10 mg of protein (corresponding to $5 \times 10^8$ HeLa cells), we obtained 10 μg (25 ng/μl) of highly purified TFIIH. This represents a 50-fold increase in the yield of recovery of TFIIH compared with the six chromatographic steps as described previously (Gerard et al., 1991).

Highly purified TFIIH was shown to exhibit several enzymatic activities (Marinoni et al., 1997; Winkler et al., 1998). As shown in Figure 1C, TFIIH(ip) possesses helicase, ATPase and CTD kinase activities comparable to the conventionally purified TFIIH(HAP). TFIIH(ip) is also able to displace the 20 nucleotide (nt) oligonucleotide from the linearized substrate (Figure 1C, Helicase, lanes 4–6), thus demonstrating the existence of a 5′→3′ XPD helicase activity. Unless gel was exposed for extended periods, XPB helicase activity was not seen, due to its very low specific activity as extensively discussed elsewhere (Coin et al., 1998; see also below, KMnO4 experiments). TFIIH(ip) possesses ATPase activities associated with the helicases, as detected by the hydrolysis of the [$\gamma$-32P]ATP (ATPase, lanes 4–6). TFIIH(ip) is also able to phosphorylate ctd4, an oligopeptide designed to mimic the CTD of the largest subunit of RNA pol II (Figure 1C, kinase, lanes 1–3). It should be noted that quantification of the ATPase, helicase and kinase activities corrected for the amount of proteins present by Western blot analysis (e.g. XPD helicase or cdk7 kinase) demonstrates that both TFIIH(ip) and TFIIH(HAP) possess the same specific activities towards the substrates tested so far.

The capacity of eluted TFIIH to function in transcription as well as in NER was also investigated. TFIIH(ip) was thus tested in a TFIIH-dependent transcription system containing highly purified recombinant TFIIB, TBP, TFIIIE and TFIIIF in addition to partially purified HeLa TFIIF and RNA pol II, using the adenosine major late promoter as a template (AdMLP). Under these conditions, TFIIH(ip) allows the same level of synthesis of a 309 nt transcript as TFIIH(HAP) (Figure 2, transcription, compare lanes 3–5 and 1–2). Moreover, TFIIH(ip) was tested for the presence of associated factors by omission of each of the basal transcription factors or RNA pol II. None of the components required for basal transcription is present in the TFIIH(ip) fraction (Figure 2, lanes 8–14).

We also tested the activity of TFIIH(ip) in an in vitro NER assay. HeLa WCE that had been previously immunodepleted of TFIIH using antibodies towards p62 subunit (3C9), was supplemented with one of the above TFIIH fractions. The TFIIH-depleted WCE exhibits a strongly reduced repair activity compared with normal WCE (Figure 2, NER, compare lanes 1 and 2). Addition of increasing amounts of TFIIH(ip) restored NER activity to a TFIIH-depleted WCE (lanes 5 and 6).

Analysis of TFIIHs from patient cell lines

Mutations in TFIIH subunits result in various phenotypes which cannot be explained on the basis of NER deficiency (Bootsma and Hoeijmakers, 1993). Having an efficient TFIIH purification procedure, we then analyzed the intrinsic activity of TFIIH from cell lines derived from different patients. Cell lines were established from several patients with genetically heterogeneous human syndromes
Impaired TFIIH function in XP patients

Fig. 1. Immunopurification of TFIIH using Ab-p44. (A) Scheme of purification of TFIIH; pep 1–17 is the peptide epitope used for specific elution. (B) The TFIIH eluted fraction TFIIH(p), (25 μl) and TFIIH(HAP) (25 μl), obtained by classical purification processes (Gerard et al., 1991), were resolved by SDS–PAGE followed either by silver staining (SDS–PAGE) or by Western blotting (WB). The nine subunits of TFIIH are indicated. (C) 1, 2 and 3 μl of immunopurified TFIIH(ip or HAP) were used to test the helicase, ATPase and kinase activities, as indicated at the top of the figure. (Δ) refers to the heated substrate and (–) to the negative control in the helicase test. 20 and 21 nt long oligonucleotide can be displaced by a 5′ to 3′ and a 3′ to 5′ helicase, respectively. For ATPase and kinase, arrows indicate the Pi generated (Pi) and the phosphorylated substrate mimicking RNA pol II CTD (ctd4), respectively. HEP is a positive control containing partially purified TFIIH.

Fig. 2. Transcription and DNA repair activities of TFIIH(ip). Upper left panel: 4 and 6 μl of TFIIH(HAP) and 2, 4 and 6 μl of TFIIH(ip) (lanes 1–5) were used in a reconstituted transcription assay containing all the basal transcription factor (except TFIIH) and the major late promoter of the adenovirus (AdMLP). Upper right panel: transcription was performed in the absence of the transcription factor as indicated at the top of the figure (lanes 6–14) and in the presence of 8 μl of TFIIH(ip). Lower panel; nucleotide excision repair (NER): 5 and 10 μl of TFIIH(ip) (lane 5 and 6) were added to a previously immuno-depleted WCE (lane 2) in the presence of an undamaged (-CDDP) or cisplatinated (+CDDP) damaged DNA. TFIIH (HAP) (5 and 10 μl in lanes 3 and 4, respectively) was used as control.

Mutation in the C-terminal domain of XPD modifies TFIIH stoichiometry

Screening of the 50 XP-D patients registered around the world (Taylor et al., 1997) reveals that most of them carry mutations in the C-terminal end of XPD, and present clinical features such as cutaneous and neurological abnormalities as well as developmental retardation. The HD2 cell line carries a R683W mutation in the C-terminal end of XPD in one of the alleles, and a L461V change as well as a 716–730 amino acid deletion (Del 716–730) in the other one (Takayama et al., 1995). However, a recent study indicated that L461V as well as Del 716–730 are null alleles and play no part in determining the phenotype in the presence of a less severe allele, R683W (Taylor et al., 1997). We then assumed that TFIIH from the HD2 cell line only contains the R683W mutant. This cell line was shown to be almost inactive in NER (Johnson et al., 1985). TFIIH [here called XPD(R683W)] from the HD2 WCE, was immunoprecipitated at low salt concentration (0.05 M KCl, washes at 0.20 M KCl), before being eluted with the epitope peptide. Although WCE protein fractions were shown to contain equivalent amounts of all the TFIIH polypeptides (Figure 3, Western blot, lanes 1 and 3), Western blotting revealed stoichiometric differences between IIH-XPD(R683W) and IIH-XPDwt immunopurified fractions (Figure 3, compare lanes 2 and 4). Indeed XPD, cdk7 and cyclin H are in lower amounts relative to the XPB, p62, p52 and p44 subunits in IIH-XPD(R683W) compared to IIH-XPDwt (Figure 3, histogram, right panel). This suggests that part of the CAK–XPD complex [the cdk activating kinase (CAK), defined as a ternary complex which contains cdk7, cyclin H and MAT1] precipitate TFIIH from HD2 WCE (a cell line derived from the XP102LO patient with mutation in XPD) or from heparin–Ultrogel 0.40 M KCl fractions obtained from WCE of SMA type I (containing only p44c), GM2252 or XPCS1BA (containing mutation in XPB) cell lines.

such as XP, or Werdnig–Hoffmann disease (the severe form of spinal muscular atrophy; SMA type I), due to mutations and/or deletions of XPD/D and p44 subunits of TFIIH, respectively (Vermeulen et al., 1994b; Bürglen et al., 1997). Ab-p44 antibody was used to immuno-
acids (Bürglen et al., 1997). Messenger RNAs from p44t and p44c genes have been detected in HeLa cells and in cells derived from SMA type II patients (J.Hoeijmakers and J.Melki, personal communications). In Werdnig–Hoffmann disease, both the p44t and the SMN genes are deleted, giving rise to TFIIH complex which only contains p44c. In addition, previous work pointed out the critical role of p44 in the TFIIH complex (Coin et al., 1998). It has been demonstrated that p44 stably interacts with XPD to stimulate its helicase activity; a failure in this interaction due to some mutations in XPD results in a decrease in XPD helicase activity. We wondered whether the differences in amino acid composition of both p44 subunits could have any consequences on the various activities of TFIIH. From the cell line of a SMA type I patient, we isolated a TFIIH containing only the centromeric form of p44 (IIH-p44c) (Figure 1). In parallel, the TFIIH from a cell line of a SMA patient with a milder form of the disease (type II) and containing both p44 genes (IIH-p44c+t) was isolated as a control.

Both IIH-p44(c+t) and IIH-p44c contain all nine subunits and reveal no stoichiometric differences in the TFIIH composition as shown by Western blotting (Figure 4, compare lanes 1 and 2). Both TFIIH fractions exhibited similar XPD helicase activity, indicating that the absence of p44t in TFIIH has no effect on the stimulation of XPD helicase activity (Figure 4, helicase). Whether p44t is present or not, the overall cdk7 kinase activity was not affected (Figure 4, kinase panels). Finally, both TFIIHs possess the same specific transcription activity towards AdML, DHFR (see lower panels) and β-globin (data not shown) promoters. Together, these data indicate that the change of three amino acids found between the p44t and p44c gene products has no effect on the overall composition, the enzymatic or transcription activities of TFIIH when tested in a basal in vitro transcription system.

**XPB mutations inhibit the transcription activity of TFIIH**

The rare XP-B patients all combine XP-B and either CS or TTD features and display a severe impairment of their NER system. Among these patients, two brothers (XPBCS1BA and XPBCS2BA) possess the same point mutation (F99S) in the N-terminal part of XPB, whereas a third one (XP11BE) carries a C→A transversion in the last intron of the XPB gene thus generating a splice mutation at the RNA level (Weeda et al., 1990; Vermeulen et al., 1994). IIH XPB(C→A) and IIH XPB(F99S) as well as their corresponding wild types (referred to herein as IIH-XPBwt1 and IIH-XPBwt2, respectively) were extracted from heparin–Ultrogel fractions as described above. SDS–PAGE followed by either silver staining or Western blotting reveals that TFIIH from patient cell lines as well as TFIIH controls have a similar subunit composition (Figure 5, SDS–PAGE and Western blot, compare lanes 1 and 2 and lanes 3 and 4). When the enzymatic activities of these TFIIHs are tested, whether or not XBP is mutated, XPD helicase and ATPase as well as cdk7 kinase remain unchanged (Figure 5). This demonstrates that mutations in XPB do not modify the three enzymatic activities associated with TFIIH. As previously demonstrated (Coin et al., 1998), it is difficult to measure the XPD helicase activity (see also below).

**TFIIHs from a patient with SMA types I or II exhibit the same activities**

In human, there are two p44 genes (p44t and p44c), which could give rise to proteins which differ by three amino acids (Bürglen et al., 1997). Messenger RNAs from p44t and p44c genes have been detected in HeLa cells and in cells derived from SMA type II patients (J.Hoeijmakers and J.Melki, personal communications). In Werdnig–Hoffmann disease, both the p44t and the SMN genes are deleted, giving rise to TFIIH complex which only contains p44c. In addition, previous work pointed out the critical role of p44 in the TFIIH complex (Coin et al., 1998). It has been demonstrated that p44 stably interacts with XPD to stimulate its helicase activity; a failure in this interaction due to some mutations in XPD results in a decrease in XPD helicase activity. We wondered whether the differences in amino acid composition of both p44 subunits could have any consequences on the various activities of TFIIH. From the cell line of a SMA type I patient, we isolated a TFIIH containing only the centromeric form of p44 (IIH-p44c) (Figure 1). In parallel, the TFIIH from a cell line of a SMA patient with a milder form of the disease (type II) and containing both p44 genes (IIH-p44c+t) was isolated as a control.

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Impaired TFIIH function in XP patients

Fig. 4. Composition and enzymatic activities of TFIIHs from patients with spinal muscular atrophy type I or II. TFIIH complexes isolated from patients with type I or II spinal muscular atrophy [IIH-p44c and IIH-p44(c+1)], respectively, were immunopurified and analyzed as described in Figure 1. Both TFIIHs were used in each enzymatic assay (1, 2 and 3 μl) and in transcription (2, 4 and 6 μl) of the MLP or DHFR promoters. Quantification of the transcription activity was done as described in Figure 3.

When tested in an \textit{in vitro} transcription assay, using AdMLP or DHFR promoters as templates, IIH(C$\rightarrow$A) and IIH (F99S) allow 15 and 60% RNA synthesis compared with the corresponding IIH-XPBwt1 and IIH-XPBwt2, respectively (Figure 5, transcription). Whereas both XPB mutations result in an almost total inhibition (~95%) of NER \textit{in vivo} and \textit{in vitro} (Weeda et al., 1990; Vermeulen et al., 1994a; Evans et al., 1997), mutation in IIH-XPB(F99S) affects the transcription capacity of TFIIH to a much lower extent than mutation in IIH-XPB (C$\rightarrow$A). This result suggests that basal transcription activity, much more than NER, is dependent on the nature of the mutation within this gene.

Open DNA promoter circumvents the effect of XPB mutations

To further investigate the effect of mutations in the XPB helicase, we took advantage of the recently described open promoter Ad(−8,+2), which was constructed following the observation that the AdMLP promoter becomes single-stranded between nucleotides −8 and +2 during the initiation step (Holstege et al., 1996). The wild-type, i.e. double-stranded AdMLP template, is transcribed only in the presence of TFIIH. Although creating a region of
Artificial DNA promoter opening abolishes the transcription inhibition due to TFIIH of XPB patient. In vitro transcription was performed from either the wild-type form (WT) or the heteroduplex (–8, +2) AdMLP promoter, in the presence of 6 μl of immunoprecipitated IIH-XPB(C→A) (A), IIH-XPB(F99S) (B) or IIH-XPD(R683W) (C) as well as from the corresponding control cell lines IIH-XPBwt1, IIH-XPBwt2 and IIH-XPDwt as indicated. Lanes 1 and 4; reconstituted transcription system lacking TFIIH. Quantification of the transcription activity was done as described in Figure 3.

heteroduplex around the start site of AdMLP Ad(–8, +2) circumvents TFIIH requirement (compare lanes 1 and 4 in Figure 6A, B and C). And synthesis of a full-length run-off transcript is notably increased upon addition of wild-type TFIIH (lanes 1 and 2 in Figure 6A, B and C; see also Dvir et al., 1997).

When added to an in vitro transcription system lacking TFIIH, IIH-XPB(C→A) and XPB (F99S) transcribe much less AdMLP than the corresponding wild-type TFIIH (Figure 6A and B, compare lanes 5 and 6 and see Figure 5). Interestingly, both IIH-XPB(C→A) and IIH-XPB(F99S) stimulate the transcription of the Ad(–8/+2) template almost to the same level (lanes 2 and 3) than their corresponding wild-type factor indicating that promoter opening is sufficient to circumvent the effect of both XPB mutations. On the contrary, whether or not XPD is mutated, both IIH-XPDwt and IIH-XPD(R683W) have the same ability to stimulate transcription (Figure 6C, compare lanes 2–3 and 4–6), suggesting that artificial promoter opening does not circumvent the effect of XPD mutation. However, IIH-XPD(R683W) stimulates transcription much less than IIH-XPDwt (Figure 3, left panel). Together, these data underline the fundamental role of XPB subunit in the transcription reaction, and especially in promoter opening.

**XPB mutation prevents promoter opening**

The above data demonstrate the strong involvement of the XPB subunit in TFIIH transcription activity. We then wondered how the XPB helicase participates in the formation of an open transcription initiation complex. Permanganate probing was used to investigate the influence of XPB mutations on the opening of AdMLP promoter. KMnO$_4$ is a chemical probe which reacts preferentially with single-stranded thymidines. The AdMLP template was incubated with a saturating amount of basal transcription factors and RNA pol II to form the preinitiation complex. Addition of both ATP and CTP leads to an enhancement of the sensitivity towards KMnO$_4$. Under these conditions, sensitivity at positions +8, +7, +5, +3, –2 and –8 designates the promoter opening around the start site (Figure 7, KMnO$_4$, lane 2 and 5).
Upon addition of IIH-XPB(C→A), KMnO₄ reacts weakly but significantly with thymidine at position +8 (lane 3), indicating first that there is promoter opening and second that the opening is not optimal (weak). On the contrary, upon addition of IIH-XPB(F99S), KMnO₄ reacts much more strongly with thymidine at positions −8, −2, +5 and +8 (lane 6) than did IIH-XPB(C→A). It is then tempting to correlate the promoter opening with the transcription activity of both mutated TFIIHs (see Figure 5). With such an hypothesis, XPB mutation could be classified as a function of its ability to open the promoter: TFIIH > IIH-XPB(F99S) > IIH-XPB(C→A).

The fact that mutated TFIIHs impede promoter opening could suggest that they cannot accurately integrate the transcription initiation complex. Knowing that the phosphorylation of the CTD of RNA pol II only occurs upon formation of the preinitiation complex including all the basal transcription factors (Lu et al., 1992) and that the subunit composition of IIH-XPB(C→A) and IIH-XPB(F99S) are stoichiometrically similar to their corresponding wild-type IIHwt1 and IIHwt2 (Figure 5), we investigated their ability to phosphorylate the CTD of the largest subunit of RNA pol II. Both IIH-XPB(C→A) and IIH-XPB(F99S) phosphorylate CTD almost to the same extent as their corresponding wild type (Figure 7, lower panel, compare lanes 2 and 3 and lanes 5 and 6) indicating first that both IIH-XPB(C→A) and IIH-XPB(F99S) accurately integrate the transcription initiation complex, and second that mutation in XPB subunit did not affect the ability of cdk7 to phosphorylate CTD.

Together these results clearly demonstrate the involvement of XPB in the opening of the promoter during transcription initiation and that phosphorylation of RNA pol II is not directly related to promoter opening.

**Discussion**

The heterogeneity of the clinical features observed among XP-B and XP-D patients was thought to be due to the involvement of XPB and XPD in a vital process of the cell, other than repair of damaged DNA. The discovery that these two helicases were part of the transcription factor TFIIH provided a rational basis for this hypothesis. This was further reinforced by studies revealing the importance of these subunits and/or their helicase activity in the initiation of protein coding genes transcription (Shaeffer et al., 1993, 1994; Guzder et al., 1994a,b). It was thus demonstrated that a natural mutation in the XPB gene, as found in one XP-B patient, resulted in a reduction of the transcription activity of the corresponding TFIIH (Hwang et al., 1996). So far, no comparative studies have been performed between different TFIIHs from XP-B or XP-D cell lines in order to define the role of both helicases in any step of the transcription reaction and to give some explanation to the diversity and the severity of the clinical features found in patients. Such studies have been largely hampered by the difficulty in obtaining sufficient quantities of highly purified TFIIH from patient cell lines. Therefore, in our investigations of the biological process which may link a mutation in either XPB, XPD or p44 to the clinical symptoms observed in patients, we developed a process that facilitates both the isolation and studies of TFIIH.

Using a two-step purification procedure including an immunoprecipitation elution, we were able to obtain highly purified TFIIH. Indeed, starting with 5x10⁶ cells, TFIIH can be purified almost to homogeneity with a 50 times higher recovery than with the classical chromatographic procedure (seven purification steps; Gerard et al., 1991), while exhibiting the same specific transcription and NER activities. Under these conditions, it is possible to obtain 10 μg (25 ng/μl) of highly purified TFIIH. This immunopurification procedure, as with those developed by others (LeRoy et al., 1998; Winkler et al., 1998), provides a quick and efficient method of purifying the various native and recombinant forms of TFIIH. It is thus possible to explore the role of XPB, XPD, cdk7 in the first steps of the transcription reaction.

**An unstable CAK–XPD complex in XPD cell lines**

The immunopurified TFIIH-XPD(R683W) partially lacks the XPB subunit and CAK complex when compared with the wild type and is accompanied by a drop in XPD helicase and cdk7 kinase activity of TFIIH. This shows that TFIIH can be resolved in vitro as well as in vivo into different subcomplexes, one of them being CAK–XPD. Addition of CAK–XPD to an in vitro transcription assay that contained all the basal transcription factors including the core TFIIH, stimulates RNA synthesis (Drapkin et al., 1996; Reardon et al., 1996; Rossignol et al., 1997). Moreover, since we know that CAK interacts fairly strongly with XPD (Rossignol et al., 1997), and that XPD interacts with p44, a subunit of the core TFIIH (Cointet et al., 1998), our results suggest that XPD most likely mediates the binding of CAK to the core TFIIH. This hypothesis is strengthened by the fact that mutations in the 3′ end of the XPD gene induced a failure in the p44–XPD interaction (Cointet et al., 1998), which would have implications on the overall TFIIH composition and activity. However, we especially noticed a 50% decrease in IIH-XPD(R683W) transcription activity which did not parallel the decrease (~80%) in both the XPD helicase and the cdk7 kinase activities. This demonstrated that both XPD and CAK are not essential for basal transcription activity, as was already shown for Rad3 in yeast genetic experiments (Sung et al., 1988, Guzder et al., 1994). The defect in transcription due to XPD mutation may reside in secondary effects, however. It is possible that the weakness of interaction observed between CAK–XPD and the core TFIIH has consequences for the optimal formation of the transcription initiation complex. Mutations in XPD may cause minor additional structural changes which could modulate its interaction with other components of basal transcription machinery.

To obtain the HD2 cell line, Johnson and collaborators fused the immortal repair-competent HeLa cell line with XP102LO, a primary fibroblast line of XP group D (Johnson et al., 1985). Further studies indicated that XP102LO has two different XPD alleles but only one (R683W) is viable in yeast (the causative allele) (Taylor et al., 1997). It is most likely that the other null allele is not present in the TFIIH complex. Our study points out that R683W mutation impairs the overall conformation of TFIIH and thus affects various enzymatic activities of TFIIH. In NER by weakening the XPD helicase activity itself, it would prevent excision of damaged oligonucleotides (Cointet et al., 1998, Evans et al., 1998). By disturbing
the interactions and the accurate contacts between CAK–XPD and the core of TFIIH when engaged in the transcription initiation complex, it will not allow optimal (and specific) RNA synthesis. These effects may include incomplete phosphorylation of transcription factor components, suboptimal positioning of the other basal or activated transcription factors. It can then be speculated that the severity of some XP-D patient phenotypes is more likely related to this small modification of the TFIIH transcriptional function than to the NER defect.

**TFIIH containing p44c exhibits activities similar to TFIIH containing p44(c+t)**

We and others demonstrated that the gene encoding p44 is duplicated in the SMA region and that one of the two p44 products (p44t) is involved in large-scale deletions associated with the most severe form of SMA, Werdnig–Hoffmann disease (van der Steege et al., 1995, Bürglen et al., 1997). The two p44 gene products differ by three amino acids. In this study we compared the composition and activity of various TFIIH complexes from patients carrying both p44 genes or those lacking the p44t gene. We found that TFIIH composition and activity was not affected by the absence of the p44t gene product in TFIIH. It is noteworthy that the XPD helicase activity is not modified. Preliminary results also indicate that the repair function of TFIIH containing only the p44c gene product is not affected in vitro, consistent with the absence of clinical symptoms related to NER defects in SMA patients. However, it cannot be excluded that the p44t gene product may have an additional activity such as a role in transcription activation of a special set of genes. A similar inhibition of specific genes encoding high sulfur proteins has been proposed to explain some of the symptoms of TTD patients. Further experiments using more specific promoters will be necessary to link definitively the severe form of SMA with deletion of the p44t gene product.

**Involvement of XPB in the opening of the promoter**

Previous observations suggested the importance of XPB, as well as its yeast homologue Rad25 in the transcription process. Indeed, yeast strains carrying the rad25 Arg-392 gene, which encodes a protein mutated in the ATP binding site, are defective in RNA pol II transcription (Park et al., 1992). The analysis of TFIIH from two XP-B patients provides insights into the role of XPB in the transcription reaction. The mutated IIH-XPB(C→A) and IIH-XPB(F99S) purified factors were shown to possess the same specific ATPase, XPD helicase and ctd kinase activities as the corresponding TFIIH wild type. However, both IIH-XPB(C→A) and IIH-XPB(F99S) exhibit reduced in vitro transcription activities by impeding promoter opening, one of the most crucial steps of transcription initiation. Indeed, permanganate probing experiments reveal a lack in promoter opening when TFIIH-XPB(C→A) is used in an in vitro transcription assay. Moreover, it has been demonstrated that recombinant XBP carrying the C→A mutation exhibits a weaker helicase activity (Hwang et al., 1996). The decrease of XBP helicase activity in TFIIH-XPB (C→A) can explain the lack in promoter opening; however, we cannot exclude the possibility that this mutation also disturbs some protein–protein interactions which take part in an optimal promoter opening. In addition, an artificially open promoter circumvents the negative effect of the XBP mutation during transcription. Interestingly, both mutations did not prevent the other enzymatic activities of TFIIH, the integration of TFIIH into the transcription initiation complex and the phosphorylation of RNA pol II. In addition, the fact that ctd phosphorylation is not connected to XBP activity suggests that this process occurs independently or before promoter opening.

Surprisingly, the C→A mutation in XBP does not affect the opening function of TFIIH in NER but prevents the formation of the 5’ incision (Evans et al., 1997), whereas the unwinding activity is significantly reduced when IIH-XPB(C→A) is used in the transcription reaction (see also Hwang et al., 1996). The apparent discrepancy between these results raised the question of the function of XBP helicase activity in NER. Although mutations in the ATP binding site of XBP inhibited the NER reaction (Park et al., 1992; van Vuuren et al., 1994), it can be speculated that, in the present case, the XBP helicase activity remaining in TFIIH-XPB(C→A) is sufficient to open DNA around the lesion but does not allow promoter opening around the transcription start site. It seems then, that this mutation in XBP highlights two roles of XBP within TFIIH: in transcription XBP function exclusively concerns promoter opening, whereas in NER it is implied in (or connected to) the 5’ incision process. It cannot be excluded, however, that XPD can partially substitute for the XBP helicase defect activity in NER but not in transcription. Whether those functions are effectively related remains to be determined.

It might appear speculative to propose a model in which XBP would be the helicase essentially devoted to transcription whereas XPD is involved in DNA repair, besides additional roles in both reactions.

**XP patients and transcription-repair syndrome**

Finally, our results revealed a relationship between the severity of the clinical features observed in XPB/CS patients and the severity of the transcription impediment. Most of the symptoms displayed by XP-CS patients, such as neurodysmyelination, retarded growth and dimorphic faces were difficult to rationalize on the basis of an NER defect. This led us to suggest that some of the symptoms observed in XP-B patients are a reflection of subtle transcription defects due to TFIIH. In this study, we clearly demonstrate a relationship between the severity of XP-B symptoms and the impairment of basal transcription for at least three different promoters. The XP-B patient with the most severe symptoms (XP11BE) is the patient with the lowest TFIIH transcription activity in vitro. Our finding points out that the severity of the clinical symptoms observed within the XP-B patients is a function of the TFIIH activity in transcription rather than in NER. Both XBP mutations result in an almost total inhibition (~95%) of NER, not only in vivo but also in vitro (Weeda et al., 1990; Vermeulen et al., 1994a; Evans et al., 1997). This observation clearly sustains the current hypothesis that XP-B and XP-D patients probably suffer from transcription repair syndromes rather than DNA repair disorders.

In addition, our study also reveals that the TFIIH extracted from an XP-D patient displays a lower transcrip-
tion activity than wild-type TFIIH. Interestingly, XP-D patients who exhibit the most severe XP phenotypes are also patients whose causative alleles are located in the 3′ end of the XPD gene (Taylor et al., 1997). These observations suggest that the transcription defect is not limited to XP-CS and XP-TTD patients but may also include XP patients, at least those carrying a 3′-end mutation in the XPD gene. It would be worthwhile correlating the nature of the mutation, the extent of the transcription defect (basal and/or activated) and the severity of the phenotypes within these groups of patients.

Materials and methods

Immunopurification of TFIIH

Whole cell extracts prepared from frozen cell pellets were fractionated on heparin–Ultrigel (Sepracor, France) equilibrated in buffer A: 0.10 M KCl (10 mM Tris–HCl pH 7.9, 20% glycerol, 0.50 mM DTT, 5 mM MgCl2). After extensive washing, proteins were sequentially eluted with buffer B/A/0.22 M, 0.40 M and 1.00 M KCl (Gerard et al., 1991). All the subunits of the core TFIIH were present in the 0.40 M KCl. This fraction was used as an input material for affinity purification. Ten milliliters of this fraction, containing ~1 mg/ml proteins, was incubated overnight at 4°C with 0.45 ml of protein A–agarose beads (Pharmacia, Sweden) crosslinked to 1H5 (Ab-p44) antibodies (Humbert et al., 1994). The resin was washed twice with buffer B (25 mM Tris–HCl pH 7.9, 20% glycerol, 0.5 mM EDTA, 0.5 mM DTT) containing 0.40 M KCl and once with buffer B containing 0.10 M KCl. The elution was then performed in 0.4 ml for 8 h at 4°C in the presence of 2 mg/ml of oligopeptide (first 17 amino acids of p44) used to obtained the 1H5 anti-p44 antibodies (Humbert et al., 1994) and 0.2 mg/ml of insulin in the same buffer. Usually a second elution was performed in a smaller volume (0.2 ml) to obtain a fraction as concentrated as the first one. The eluted fractions were dialysed twice in buffer B containing 0.05 M KCl to eliminate the peptide. We then obtained 10 μg (25 ng/μl) of highly purified TFIIH. This immunopurification procedure can be used with any human cells; the smallest amount of cells we used was ~1–3×106 cells.

Cell strains and culture conditions

Human fibroblasts cultures CSRO (control) and XPCS1BA were grown in Ham's F10 medium supplemented with 11% fetal calf serum (FCS) and antibiotics. Human lymphoblastoid cells GM2252 (derived from XP11BE patients), GM1855 (derived from the patient mother) and cell lines derived from SMA type 1 or II patients (Bürglen et al., 1997) were grown in suspension in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% FCS. Cell lines N2 with (XP102LO) and HeLa were grown in Dulbecco's medium supplemented with 5% FCS and antibiotics.

In vitro transcription and NER assays

The reconstituted transcription assay contained purified transcription factors, TBP, TFIIA, TFIIB, TFIIE, TFIIH, TFIIF and RNA pol II was performed as described (Gerard et al., 1991). Briefly, the eluted fraction containing TFIIH was incubated with the transcription factors and 100 ng of the AdMLP template (AdMLP) was incubated with the transcription factors and 100 ng of the AdMLP template (EcoRI–SalI) in 50 mM Tris–HCl pH 7.9 buffer containing 10% glycerol, 1 mM EDTA, 0.5 mM DTT and 5 mM MgCl2. Reaction mixtures (final volume = 20 μl) were incubated for 15 min at 25°C to allow the formation of the preinitiation complex. Transcription was then initiated by addition of the transcription template which was purified by a selective ligation at 20°C for 5 min and finally heated at 55°C for 30 min. After cooling, circular AdMLP was circularized with SalI. Run-off transcription of this template gives rise to a 290 nt transcript.

KMMo sensitivity assay

AdMLP template (20 ng) was incubated at 25°C for 30 min with recombinant TBP, TFIIA, TFIIB, TFIIH, highly pure pol II and TFIIH as indicated, in a 20 μl reaction that contained 50 mM Tris–HCl pH 7.9, 10% glycerol, 1 mM EDTA, 0.5 mM DTT, and 5 mM MgCl2. ATP and CTP (200 μM) were added for the last 5 min. Two microlitres of the 160 μM KMMo was added for 2 min, after which the reaction was stopped by addition of 2 μl of 14.4 M β-mercaptoethanol. After phenol–chloroform extraction, DNA was recovered by ethanol precipitation, redissolved in water and subjected to 30 cycles of primer extension using an end-labelled primer. After phenol–chloroform extraction, ethidium precipitation and wash, the sample was loaded onto a 6% sequencing gel. The gel was dried and autoradiographed.

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Cointet al.


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