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

Frédéric Coin, Etienne Bergmann, Alexandre Tremeau-Bravard and Jean-Marc Egly[1]

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, B.P.163, 67404 Illkirch Cedex, C.U. de Strasbourg, France

[1]Corresponding author
e-mail: egly@igbmc.u-strasbg.fr

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 (Bürglen *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 XPB and XPD mutations in transcription and especially in promoter opening, using artificial premelted promoter and $KMnO_4$ footprinting experiments. Together, our results point out the crucial role of XPB 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 millilitres 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 5×10^8 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 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, $KMnO_4$ experiments). TFIIH(ip) possesses ATPase activities associated with the helicases, as detected by the hydrolysis of the [γ-^{32}P]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, TFIIE and TFIIF in addition to partially purified HeLa TFIIA and RNA pol II, using the adenovirus 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

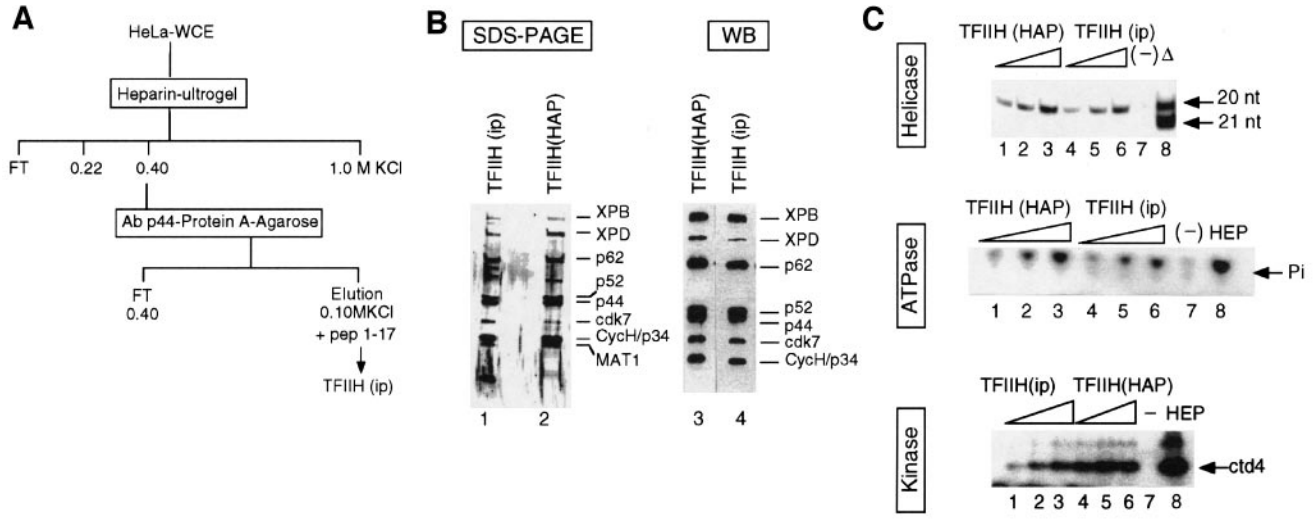

Fig. 1. Immunopurification of TFIIH using Ab-p44. (**A**) Scheme of purification of TFIIH; pep 1–17 is the peptide epitope used for specific elution. (**B**) The TFIIH eluted fraction TFIIH(ip), (25 µl) and TFIIH(HAP) (25 µl), obtained by classical purification processes (Gerard *et al*., 1991), were 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 immunopurifed 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.

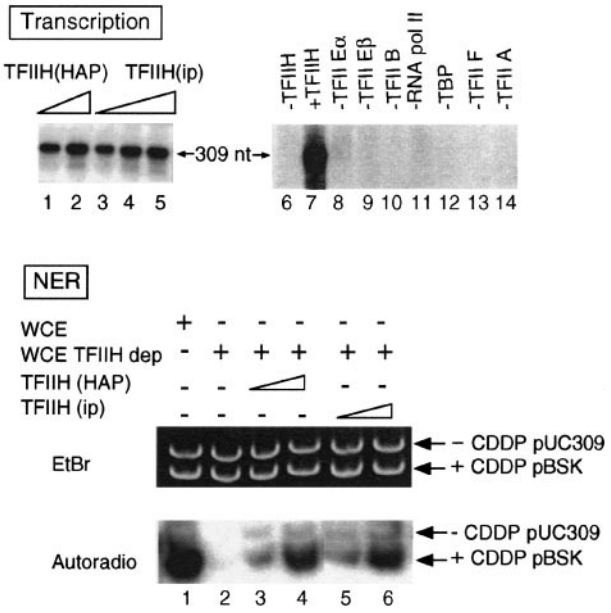

Fig. 2. Transcription and DNA repair activities of TFIIH(ip). Upper left panel: 4 and 6 µl of TFIIH(HAP) and 2, 4 and 6 µl of TFIIH(ip) (lanes 1–5) were used in a reconstituted transcription assay containing all the basal transcription factor (except TFIIH) and the major late promoter of the adenovirus (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 immunodepleted 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.

such as XP, or Werdnig–Hoffmann disease (the severe form of spinal muscular atrophy; SMA type I), due to mutations and/or deletions of XPB/D and p44 subunits of TFIIH, respectively (Vermeulen *et al*., 1994b; Bürglen *et al*., 1997). Ab-p44 antibody was used to immunoprecipitate 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.

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]

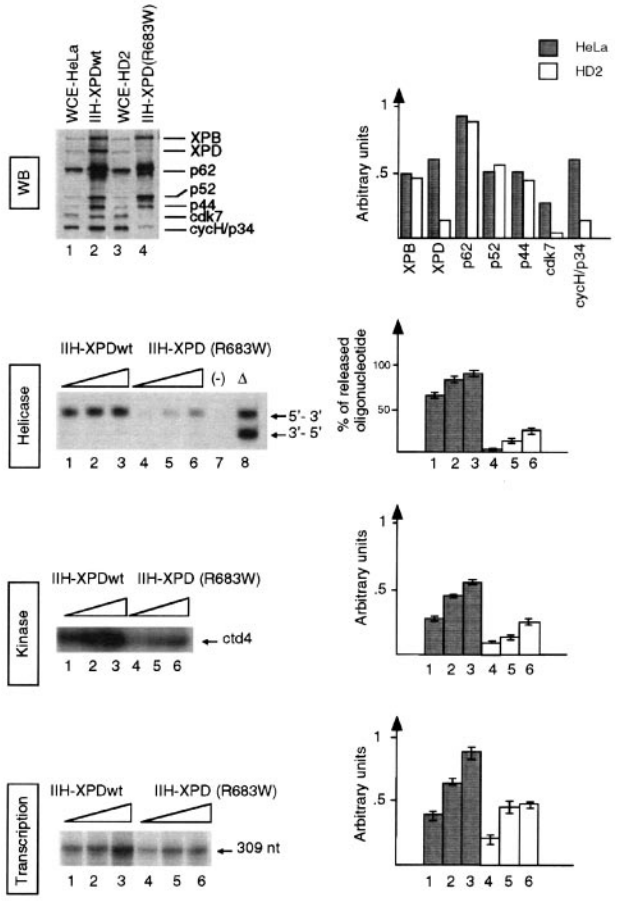

Fig. 3. Composition and enzymatic activities of TFIIH isolated from HD2 cells containing a mutation in the XPD gene. WCE from HeLa or HD2 cell lines (lanes 1 and 3) as well as TFIIH complexes called IIH-XPD wt and IIH-XPD (R683W) (lanes 2 and 4, respectively) were resolved by SDS–PAGE followed by Western blotting (WB) using antibodies towards the different subunits of TFIIH. IIH-XPD(R683W) and IIH-XPDwt were also assayed in helicase and kinase (1, 2 and 3 μl) as well as transcription (2, 4 and 6 μl). Right panel: quantification of the polypeptide compositions as well as the enzymatic and transcription assays was done using a Bio-imaging analyser. The means ± SE of three independent experiments are indicated.

was dissociated from the core IIH-XPD(R683W) in our experimental conditions. To perform the various enzymatic assays, TFIIH concentration was estimated by Western blotting and adjusted to equivalent values of the subunits of the core TFIIH (XPB, p62, p52 and p44). IIH-XPD(R683W) exhibits a weaker XPD helicase and kinase activity than the IIHwt (Figure 3, helicase, compare lanes 4–6 with lanes 1–3; kinase, compare lanes 4–6 with lanes 1–3), confirming the absence of XPD and cdk7.

Interestingly, the transcription activity of IIH-XPD(R683W), although weaker than the corresponding wild type, is not directly proportional to either the XPD helicase or the cdk7 kinase activities (Figure 3, see histograms of helicase, kinase and transcription activities, right panel).

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.

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*., 1994a). 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 TFIIH are tested, whether or not XPB 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 XPB helicase activity (see also below).

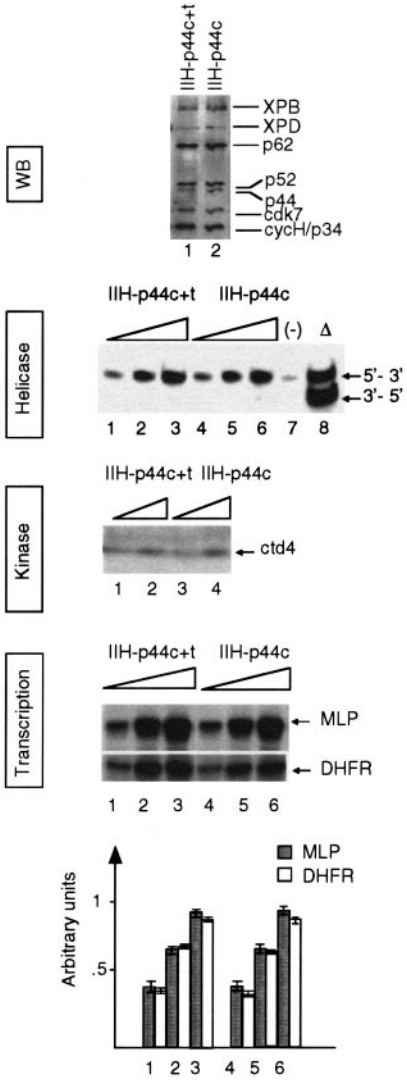

Fig. 4. Composition and enzymatic activities of TFIIHs from patients with spinal muscular atrophy type I or II. TFIIH complexes isolated from patients with type I or II spinal muscular atrophy [IIH-p44c and IIH-p44(c+t), respectively] were immunopurifed and analyzed as described in Figure 1. Both TFIIHs 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.

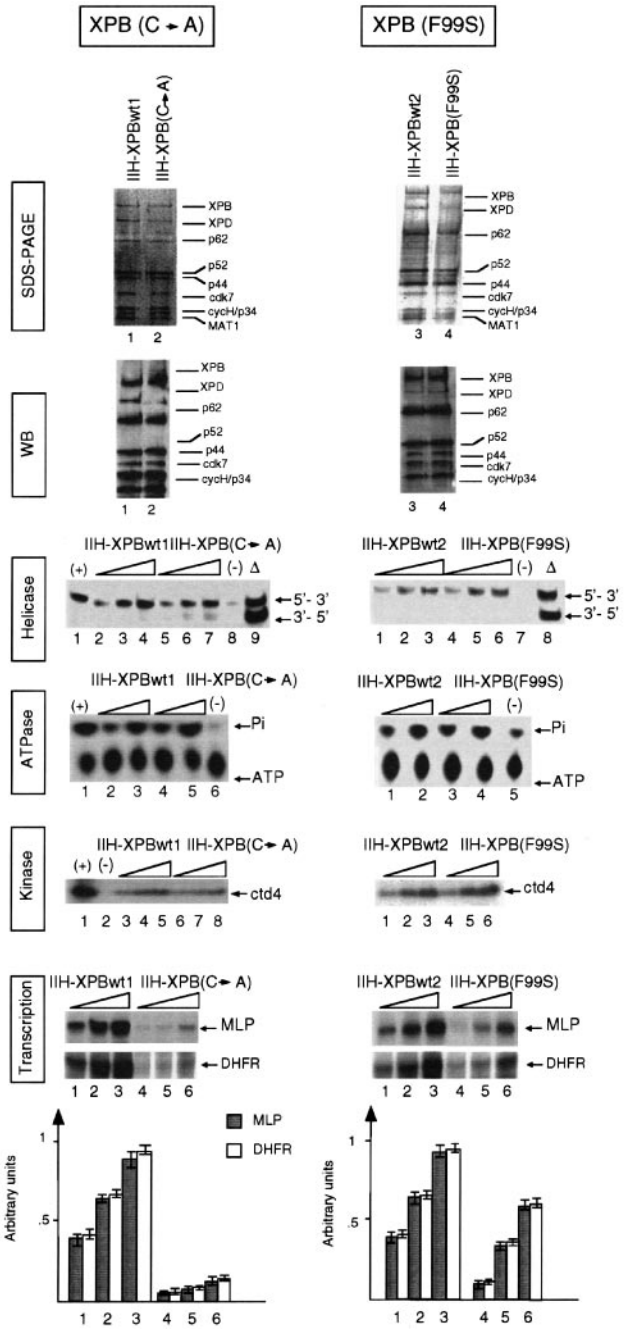

Fig. 5. Mutations in the XPB subunit differentially impede TFIIH transcription activity. TFIIH complexes from GM2252 [IIH-XPB(C→A), left panel] or XPBCS1BA [IIH-XPB(F99S), right panel] cell lines as well as their wild-type counterparts from GM1855 or CSRO well lines (IIH-XPBwt1 and IIH-XPBwt2) of XPB were immunopurified and resolved by SDS–PAGE following by Western blotting (WB) before being tested in various assays as indicated at the left of each panel. The four TFIIH were used in each enzymatic assay (1, 2 and 3 μl) and in transcription (2, 4 and 6 μl) of the MLP or DHFR promoter. Quantification of the transcription activity was done as described in Figure 3.

When tested in an *in vitro* transcription assay, using AdML or DHFR promoters as templates, IIH(C→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 *in vivo* and *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→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

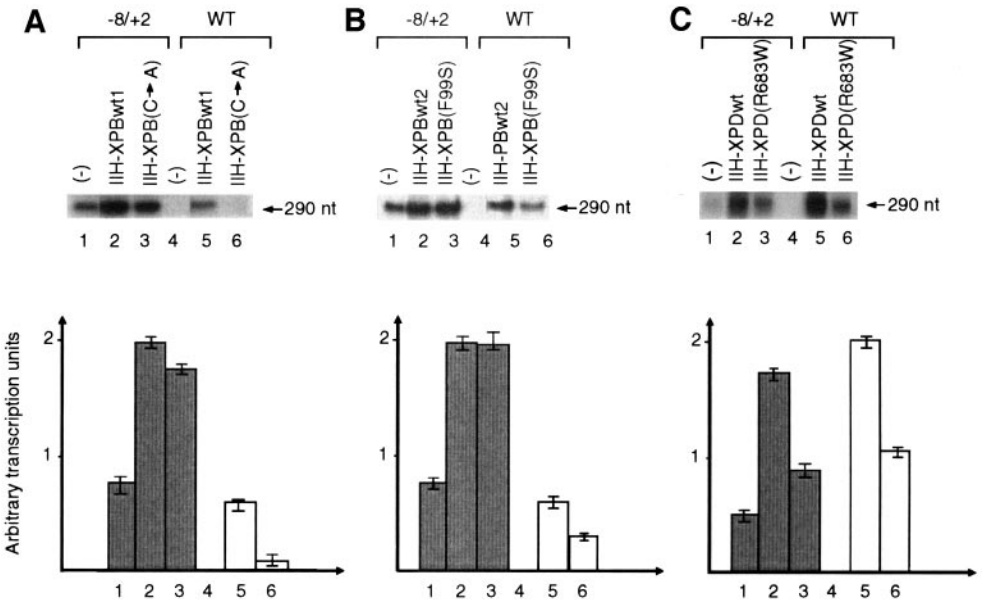

Fig. 6. Artificial DNA promoter opening abolishes the transcription inhibition due to TFIIH of XPB patient. *In vitro* transcription was performed from either the wild-type form (WT) or the heteroduplex (–8,+2) AdMLP promoter, in the presence of 6 µl of immunoprecipitated IIH-XPB(C→A) (**A**), IIH-XPB(F99S) (**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

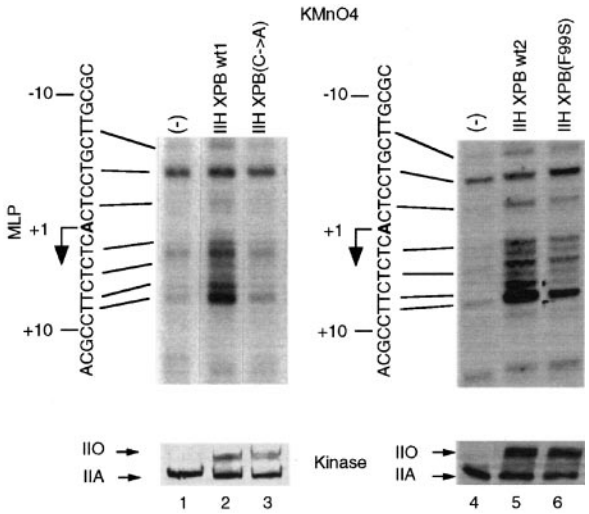

Fig. 7. XPB mutations prevent promoter opening. $KMnO_4$ sensitivity assay of the non-template strand of the AdMLP promoter was performed using 6 µl IIH-XPBwt1, IIH-XPB(C→A), IIH-XPBwt2 and IIH-XPB(F99S) in a system containing all the basal transcription factors except TFIIH, as indicated in Materials and methods. Depicted to the left of the autoradiogram is part of the sequence of the non-template strand of the AdMLP. The arrow indicates the start site and direction of transcription. Hypersensitive sites are indicated. Lower panel: the phosphorylation of RNA pol II was tested in parallel in an *in vitro* transcription assay containing all the basal transcription factors and the various TFIIH as indicated at the top of the panel. Quantification of the hyperphosphorylated RNA pol II (IIO) was performed by Western blotting.

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), $KMnO_4$ 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), $KMnO_4$ 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 5×10^8 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 XPD 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 (Coin *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 (Coin *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 (Coin *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 p44 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 XPB carrying the C→A mutation exhibits a weaker helicase activity (Hwang *et al*., 1996). The decrease of XPB 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 XPB 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 XPB activity suggests that this process occurs independently or before promoter opening.

Surprisingly, the C→A mutation in XPB 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 XPB helicase activity in NER. Although mutations in the ATP binding site of XPB inhibited the NER reaction (Park *et al*., 1992; van Vuuren *et al*., 1994), it can be speculated that, in the present case, the XPB 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 XPB highlights two roles of XPB within TFIIH: in transcription XPB 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 XPB 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 XPB 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 XPB 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 extend 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–Ultrogel (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 $MgCl_2$). After extensive washing, proteins were sequentially eluted with buffer 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 millilitres 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 ~$1–3\times10^7$ cells.

Cell strains and culture conditions

Human fibroblast 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 I or II patients (Bürglen *et al*., 1997) were grown in suspension in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% FCS. Cell lines HD2 (derived from 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 containing 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 (*Eco*RI–*Sal*I) in 50 mM Tris–HCl pH 7.9 buffer containing 10% glycerol, 1 mM EDTA, 0.5 mM DTT and 5 mM $MgCl_2$. 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 NTPs including [α-^{32}P]CTP (400 µCi/mmol). The transcription was carried out for 45 min at 25°C. RNA transcripts were resolved by electrophoresis and analyzed by autoradiography.

For NER reaction, WCEs were prepared from HeLa cells. Reactions contained 250 ng of pUC309 DNA plasmid randomly damaged with *cis*-diamino-dichloro-platin and an equal amount of undamaged pSK plasmid as an internal control. The reaction was carried out as described (Winkler *et al*., 1998). Briefly, TFIIH-depleted WCE was supplemented with purified TFIIH as indicated and mixed with DNA substrates for 3 h at 30°C in the presence of [α-^{32}P]dATP. DNA was then purified, linearized with *Eco*RI and analyzed on a 0.8% agarose gel. HeLa WCE was depleted of TFIIH after incubation overnight at 4°C with antibodies towards p44 crosslinked to protein A.

TFIIH enzymatic assays

The helicase substrate was obtained by annealing 5 ng of an oligonucleotide corresponding to the fragment 6219–6255 of single-stranded M13mp18 (–) DNA, to 1 µg of single-stranded M13mp18. The resulting heteroduplex was digested for 1 h at 37°C with *Eco*RI (New England Biolabs) and then extended to 21 and 20 bp, respectively, with the Klenow fragment (5 units) in the presence of 50 mM dTTP and 7 µCi [α-^{32}P]dATP (3000 Ci/ mmol, Amersham). Helicase assay was then performed as described (Coin *et al*., 1998).

The enzymatic hydrolysis of ATP was assessed as previously described (Roy *et al*., 1994b). Briefly, protein fractions were incubated for 2 h at 30°C in the presence of 1 µCi [γ-^{32}P]ATP (7000 Ci/mmol, ICN Pharmaceuticals) in a 20 µl reaction volume in 20 mM Tris–HCl pH 7.9, 4 mM $MgCl_2$, 1 mM DDT, 50 µg/ml BSA. Reactions were stopped by adding EDTA to 50 mM and SDS to 1% (w/w). The reactions were then diluted 5-fold, spotted onto polyethylenimine (PEI) TLC plates (Merck), run in 0.5 M LiCl/1 M formic acid and autoradiographed.

Kinase assays were carried out in a 20 µl reaction volume containing 20 mM Tris–HCl pH 7.9, 7 mM $MgCl_2$, 0.5 mg/ml BSA, 30 mM KCl, 1 µg of ctd4 (a synthetic tetrapeptide of SPTSPSY), 2.5 µCi of [γ-^{32}P]ATP. Samples were incubated 30 min at 25°C and reactions were stopped by the addition of 3 µl of loading buffer. After SDS–PAGE (15%), the gel was fixed and dried on Whatman filter paper. The phosphorylated ctd4 was visualized by autoradiography.

RNA pol II phosphorylation was carried out as a classical run-off transcription except that ATP was added to 5 mM final concentration. The reactions were stopped by addition of 20 µl of Laemmli buffer and samples were loaded on a 5% SDS–PAGE. The polymerase polypeptides were revealed using the 7C2 antibody (Besse *et al*., 1995).

Formation of Ad(–8/+2) template

For the production of the heteroduplex AdMLP (–8/+2) template, two different double-stranded oligonucleotides (5′-AATTCCCTATAAAAGGGGGTGGGCGCGCGT**AGCAGGAGTG**TCTCTTCCTCG-3′ and 5′-AATTCCCTATAAAAGGGGGTGGGCGCG CGT**TCGTCCTCAC**-TCTCTTCCTCG-3′) with an inverted region (bold sequence), from –8 to +2 nt of the transcription start site, were cloned in the *Eco*RI–*Bam*HI sites of the pUC309 plasmid, replacing the wild-type AdMLP promoter. Each resulting plasmid was then digested with *Eco*RI or *Hin*dIII, respectively, and the two linear molecules were then mixed in a reaction mixture (at a concentration of 20 ng/µl) containing 10 mM Tris–HCl, 10 mM NaCl and 1 mM EDTA and denatured for 15 min at 95°C. The mixture were then placed on ice, and after addition of 150 mM NaCl final, heated at 55°C for 30 min. The reaction mixture was then slowly cooled down at room temperature to allow rehybridization of DNA molecules. Half of the DNA molecule forms the expected heteroduplex template which was purified by a selective ligation at 20°C for 5 min and a CsCl gradient. After purification, circular Ad(–8/+2) was linearized with *Sal*I. Run-off transcription of this template gives rise to a 290 nt transcript.

$KMnO_4$ sensitivity assay

AdMLP template (20 ng) was incubated at 25°C for 30 min with recombinant TBP, TFIIB, TFIIF, TFIIE, 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 $MgCl_2$. ATP and CTP (200 µM) were added for the last 5 min. Two microlitres of the 160 mM $KMnO_4$ 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, ethanol precipitation and wash, the sample was loaded onto a 6% sequencing gel. The gel was dried and autoradiographed.

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