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p44/SSL1, the regulatory subunit of the XPD/RAD3 helicase plays a crucial role in the transcriptional activity of TFIIH.

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Abstract

In order to unravel the mechanism that regulates transcription of protein coding genes, we investigate the function of the p44 subunit of TFIIH a basal transcription factor which is also involved in DNA repair. We previously have shown that mutations in the carboxy terminal of the XPD helicase, another subunit of TFIIH, prevent its regulation by p44 (Coin et al., 1999). Using a site directed mutagenesis approach within the p44 region from aa 66 to aa 200, we evidence how a decrease in the interaction between p44 and XPD results in a decrease of the XPD helicase activity and leads to a defect in the first steps of the transcription reaction, namely the first phosphodiester bond formation and promoter clearance. We thus provide some explanation for the transcriptional defect found in SSL1 mutated yeast (Wang et al., 1995). Moreover, this study evidences how the activity of the CAK complex (the cdk activating kinase associated with TFIIH) in stimulating transcription is mediated in part by p44/XPD interaction.
Introduction:

TFIIH is a multisubunit complex involved in two major DNA metabolism pathways, transcription and nucleotide excision repair (1). The transcriptionally active form of TFIIH, also called holo-TFIIH, includes core TFIIH, a five subunit subcomplex constituted of XPB, p62, p52, p44 and p34, as well as XPD and the three subunits of the cyclin-dependent kinase activating kinase complex (known as CAK): cdk7, cyclin H and MAT1. Mutations in \textit{XPB} and \textit{XPD} genes, encoding the two DNA-dependent ATPase associated helicases (2-7) are responsible for several rare autosomal recessive human genetic disorders, including \textit{xeroderma pigmentosum}, Cockayne syndrome and trichothiodystrophy (8-11).

Investigations on cells derived from XP-B and XP-D patients showed a significant decrease of both transcription and NER activities (12, 13). Many XP-B and XP-D patients suffer from a high UV-sensitivity due to the inability of their NER machinery to remove DNA damages (14, 15). The NER reaction is dependent on both helicases, XPB and XPD, that play an essential role in the formation of an open complex structure necessary for the subsequent incisions on each side of the lesion by the site-specific endonucleases, ERCC1-XPF and XPG (1, 16-18).

In transcription, TFIIH is involved at different levels of the initiation step. A mutation in the ATP binding domain of XPB gives rise to a transcription defect due to an impaired promoter opening thus preventing the synthesis of the first phosphodiester bond by RNA polymerase II (RNA pol II) (19-22). The role of the XPD helicase, although rather elusive, appears to be less essential for the transcription initiation process. The XPD helicase activity is not required for both promoter opening and first phosphodiester bond synthesis, nevertheless it significantly stimulates transcription. Indeed a mutation in the ATP binding site of XPD strongly diminishes the \textit{in vitro} RNA synthesis (4, 20, 23). In addition, the physical presence of XPD is required for an efficient promoter escape (22).

It has recently been shown that the p44 subunit of core TFIIH interacts with and strongly stimulates the XPD helicase activity (24). p44 although it does not possess any enzymatic activity, plays therefore an essential role within TFIIH. In addition, the C-terminal end of p44 contains a zinc finger and RING finger-like domains, the role of which is not yet elucidated. Unlike the case with \textit{XPD} and \textit{XPB} genes, no human genetic disorders have been associated with the gene encoding p44.
However, large scale deletions found in the most severe type of spinal muscular atrophy (SMA type I) known as Werdnig-Hoffmann disease often implicate the telomeric p44 gene in addition to the SMN and NAIP genes (25). Only a few mutations of the SSL1 gene, the yeast counterpart of p44, have been found, pointing out the probable lethality of the large majority of Δssl1 mutations (26, 27). These mutants can perform RNA pol II-dependent transcription at permissive temperatures but this activity is abolished upon preincubation of yeast extracts at a restrictive temperature. Unlike transcription, a NER defect was observed even at permissive temperatures, explaining the constitutive UV-sensitivity of these cells. The NER defect associated with UV-sensitivity was also found in XP-D patients bearing a mutation in the C-terminal part of the XPD protein. This results in an absence of interaction between p44 and XPD and in an inhibition of the XPD helicase activity (24).

In the present study, we show that mutations in the N-terminal part of the human p44 prevent the XPD/p44 interaction leading to a significant diminution of transcription. We demonstrate that the recombinant TFIH carrying such p44 mutations is deficient in the first steps of the transcription initiation reaction: the first phosphodiester bond formation and promoter clearance. The consequences of such mutations may explain the transcription defect observed in Δssl1 mutant yeast.
Materials and Methods

Construction of recombinant baculoviruses expressing TFIIH subunits

Baculovirus expressing a single TFIIH subunit were constructed in the pVL1392 or pACAB4 vectors (PharMingen). The cDNAs coding for XBP, XPD, XPDG675R, p62, p52, p34, cdkt7, cyclin H and MAT were inserted into the pVL1392 expressing vector as previously described (20, 24, 34). Each p44 mutant was obtained by PCR-mutagenesis using to pairs of oligonucleotides, GGAATTCCATGGATGAAGAACCTGAAAGAACT in combination with either GTATCTGGTTATTCGATTGCTGCAGAA, CTTACAACCTTGCCGCCCCATCTAATATT, CATAAAGTCAATGCAGAATTACATC, TTTGATCAAAATGCGCCATTAGTCAGATT, TATGTGCTAGTACGGCATGAAAGAACA or GGACATACAAGGCGCAAGTACTAATC (to synthesize the 5’ part of p44G200R, D178A, E166A, P100A, D66A and R165A respectively) and CGGATCCAACACCTGAAGGAGCTGGAATCTT in combination with either TCTGCAGACATGCTTGCCATAACAGACAC, TATTAGATGGGCAGCAAGTTGTAAG, GATGATTAGTACTCGACTTGTATG, AATCTGACTAATGGCATTTTGATCAAA, TGTTCTTATGATCAGTACTCACCACA or GATTAGTACTTCCGACTTGTATGTC (to synthesize the 3’ remaining part of p44G200R, D178A, E166A, P100A, D66A and R165A respectively). Wild type as well as mutant p44 cDNAs were inserted at EcoRI/BamHI sites. Both XBP and cyclin H are fused to a 6-histidine tag at their N-terminal extremity. The resulting vectors were recombined with baculovirus DNA (BaculoGold DNA, PharMingen) in Sf9 cells (Spodoptera frugiperda). The recombinant viruses were purified from isolated plaques, and viral stocks were prepared by a three-step growth amplification. The calculation of the MOI of each virus stock solution as well as the determination of the best ratio between the expression of either subunit allowed us to optimise the co-production of the different proteins in a rather stoichiometric pattern.

Purifications of TFIIH complexes

Cobalt chelate affinity purification: Sf9 cells are infected with combinations of baculoviruses expressing the different subunits of TFIIH as previously described (20). 48 hr after infection, cells were collected washed once and dounced in lysis buffer (20 mM Tris-HCl pH 7.8, 20% glycerol, NaCl 150 mM, 0.1% Nonidet P40, 5 mM β-mercaptoethanol, 0.5 mM PMSF, 1x protease inhibitor cocktail). DNA as well as cell membranes were pulled down by centriguation at 14000 g during 30
min (34). The supernatants were first applied on a heparin ultrogel column. After a five-resin volume wash with buffer A containing 0.4 M NaCl, the proteins were eluted with five volumes of resin of the same buffer containing 0.5 M NaCl. After a 3 hr dialysis against buffer B (50 mM Tris-HCl pH 7.8, 20% glycerol, 300 mM KCl) fractions were incubated for 1 hr at 4°C with 1/40 fraction volume of cobalt chelate affinity resin (Talon, Clontech). After a 20-volume wash with buffer B containing 10 mM imidazol, proteins were eluted in buffer B containing 100 mM EDTA and dialysed against buffer C (50 mM Tris-HCl pH7.8, 20% glycerol, 0.1 mM EDTA, 0.5 mM DTT and 50 mM KCl).

Immunoaffinity purification: 0.5 M NaCl heparin fractions were dialysed against buffer C and incubated with anti-p44 antibodies crosslinked to protein A-sepharose beads for 12 hr at 4°C buffer C containing 0.1% Nonidet p40. After three washes with buffer C containing 300 mM KCl in addition to 0.1% Nonidet P40, proteins were eluted in one beads volume of buffer C containing 2 mg/ml of epitope peptide during 24 hr.

Abortive initiation assays
These reactions were carried out as previously described (22). Briefly, preinitiation complexes were assembled using AdMLP template, TBP, TFIIB, TFIIE, TFIIF, TFIIH from various sources and RNA polymerase II in the presence of 0.4 mg/ml BSA and 5 mM MgCl₂ at 28°C during 30 minutes. Phosphodiester bond synthesis was initiated by addition of CpA (0.5 mM), (³²P)α-CTP and dATP (4 µM) plus MgCl₂ to 6.5 mM. After 30 minutes for synthesis of the trinucleotide, the reactions were stopped by the addition of 100 mM EDTA and 0.5 mg/ml proteinase K. The samples were then applied on a 20% polyacrylamide 8.3 M urea gel and run at 20 W.

Promoter escape reactions
These reactions were carried out as previously described (22). Typically, preinitiation complexes were assembled in the same conditions as in the abortive initiation reaction but in the presence of a premelted AdMLP (-8/+2) heteroduplex template. In order to initiate transcription in the absence of ATP, priming dinucleotides ApG were used as initiating substrate. After 30 minutes of incubation for PIC formation, we started transcription by adding CTP and GTP to 5 µM, (³²P)α-UTP, cordycepin to 100 µM, dATP to 4 µM and MgCl₂ to 6.5 mM. After 30 minutes at 28°C the
reactions were stopped by the addition of 30 mM EDTA and 0.1 mg/ml proteinase K and samples were applied on a 20% polyacrylamide gel, 8.3 M urea and run at 20 W.

RESULTS

Mutations in the N-terminal part of p44 abolishes its regulatory function towards the XPD helicase.

Having previously shown that the N-terminal portion of p44 interacts with the C-terminal part of XPD (24), we investigated the consequences of mutations in p44 on TFIIH activities. We performed a multiple sequence alignment of the N-terminal part of p44 from different organisms to determine the localization of the most conserved regions (accession numbers: human p44: Z30094, drosophila: AC005720, C. elegans: Z30662, S. pombe: c1682, S. cerevisiae: 1360294, A. thaliana: AC005322). Given that this region of p44 does not contain any consensus motif potentially essential for the function of the protein, we generated several mutated recombinant TFIIHs by introducing point mutations in the most conserved codons of the 5’ end of p44. Five of the six designed recombinants contain mutations that correspond to a conversion of a conserved amino acid into an alanine (Figure 1; D66A, P100A, R165A, E166A, D178A). The sixth mutation mimics the G to R transversion already reported in the yeast SSL1 gene, the counterpart of the human p44 (27). These point mutations are expected to affect various types of predicted secondary structures of p44 (β-sheet: D66A, R165E, E166A; α-helix: D178A and coil: P100A, G200R) (29). The mutations have been generated by PCR, following a classical procedure of site directed mutagenesis. PCR products were subcloned into pVL1392 vector and finally inserted into the baculovirus genome by recombination. The cDNAs coding for cyclin H and XPB have been fused to a His-tag coding sequence at the 5’ extremity. Sf9 insect cells were thereafter infected with the recombinant viruses in order to produce either p44 alone, TFIIH subcomplexes (IIH6, containing core TFIIH plus XPD) or holo-TFIIH (IIH9, containing the nine subunits) complexes, that were then further purified by cobalt chelate affinity (20). Given that the recombinant p44 protein is not fused to a His-tag, this subunit alone was purified by immunoaffinity and subsequently eluted using an epitope peptide (13).
To investigate if mutations within p44 are detrimental for XPD helicase activity, we first tested the ability of XPD to interact with p44. Therefore, we carried out immunoprecipitations on crude extracts of Sf9 cells infected with baculoviruses overexpressing XPDwt and either p44G200R, p44D178A, p44E166A, p44P100A, p44D66A or p44165A using monoclonal antibodies raised against XPD. After extensive washing at 0.4M KCl, the immunoadsorbed proteins were analyzed by SDS-PAGE followed by western-blotting analysis. XPDwt exhibited an interaction with p44P100A and p44R165A as strong as with p44wt (Figure 2A, compare lanes 6 and 8 with lane 1), whereas it did not bind p44G200R and p44D66A (lanes 3 and 7). As a control we used the recombinant XPD-G675R reproducing the transversion G into R of the residue 675 of XPD that was reported in a human XP-D patient (30). As already shown (24), the overexpressed XPD-G675R subunit did not interact with p44wt (lane 2). Furthermore, we observed a weak interaction between either p44D178A or p44E166A and XPDwt polypeptides (lanes 4 and 5). It should be noticed however, that the differential complex formation was not simply a result of variable steady state levels of expression of either wild type or mutant p44 (Figure 2A, compare load and IP). In another set of experiments, we investigated the consequences of p44 mutations on the XPD helicase activity. XPD/p44 complexes immobilised by antibodies raised against XPD (see Materials and Methods) were then tested for their ability to unwind the DNA using an assay in which the displacement of an oligonucleotide from the M13 single-stranded plasmid was measured. p44wt bound to XPD stimulated XPD helicase activity up to 5-6 fold (Figure 2B, lanes 2 and 3). We noticed that the XPD helicase activities are roughly proportional to the amount of p44 with which it co-immunoprecipitates. p44P100A and p44R165A interacted with XPD and stimulated its 5’ to 3’ helicase activity as observed with p44wt (Figure 2B; compare lanes 7 and 9 with lane 3). On the contrary, although XPD was overexpressed with either p44G200R or p44D66A, it did not coimmunoprecipitate with these two recombinant p44 polypeptides and was not stimulated by any of them; in such cases, XPD displayed the same 5’->3’ helicase activity as XPDwt alone (compare lanes 4 and 8 with lane 2). An intermediate pattern of helicase activity was repeatedly observed with the p44D178A and the p44E166A XPD partners (Figure 2B, lanes 5 and 6). Interestingly, mutations affecting two successive residues of p44 (R165A and E166A) led to very different helicase activities. Indeed, the p44R165A/XPDwt heterodimer was fully active in helicase assays, whereas
p44E166A/XPDwt exhibited a weak helicase activity. Together our data show that the 5'-3' XPD helicase activity and XPD/p44 interaction are qualitatively and quantitatively connected, pointing out the important role of p44 in the regulation of the XPD helicase activity.

As we previously mentioned, the 5'-3' helicase activity of XPD is dependent on both the presence of DNA and the ATP hydrolysis. Assuming that the absence of XPD/p44 interaction might affect the XPD ATPase activity required for DNA unwinding, we were interested in measuring this activity when XPD is associated with p44. For this purpose, we ran an ATPase assay as described by Roy and co-workers (32), in which immunoprecipitated XPD containing fractions (i.e. XPD/p44 heterodimer) were incubated with γ-32P(ATP) as a substrate, in the presence of DNA. We observed that none of the mutated recombinant p44 modulate the XPD ATPase activity. Whether p44 is present or not, the level of hydrolysis of ATP by XPD remains constant (Figure 2C compare lanes 5-10 with lanes 3 and 4). Moreover, incubation of the substrate with increasing amount of proteins revealed that the ATP hydrolysis has not gone to saturation and is not affected by mutations within p44 (Figure 2D compare lanes 2-4 with 5-7 and 8-10). Therefore, although the XPD helicase activity drops in the presence of some of the mutated p44 recombinants, the ATP hydrolysis remains unchanged, meaning that the ATPase activity is independent of the helicase even if the helicase requires ATP hydrolysis for its DNA unwinding activity.

**Mutations in p44 affect the transcriptional activity of TFIIH**

We then investigated the effect of p44 mutations on the transcriptional activity of the IIH6 recombinant complexes that contain 5 subunits of the "core TFIIH", including either the wild type or the mutated p44 subunit, in addition to XPDwt or XPDG675R. In this case, XPD was similarly expressed (Figure 3A, compare the load, XPD and p62). The different complexes overexpressed in Sf9 cells were purified on a heparin Ultrogel column, the 0.5 M KCl eluted fraction of which was loaded subsequently on a cobalt chelate affinity column to retain the His-tagged XPB containing TFIIH complexes (20). p44 is a central protein in the core TFIIH complex and has been shown to interact with XPB, p62 and p34 (31 and our unpublished results). We then tested by immunoprecipitation the interactions between each of these three polypeptides and the various p44 mutants. The three TFIIH subunits were able to coprecipitate with the different p44 mutants, meaning that the different designed mutations do not affect the interactions between p44 and its
three partners within core TFIIH (data not shown). The composition of each recombinant IIH6 complex was analysed by western blotting (Figure 3A). We noticed variations in the amount of XPD associated with the different complexes (Figure 3A: compare lanes 3, 4 and 7 with lane 6). The quantity of XPD within the complex was roughly proportional to the strength of the interaction between p44 and XPD (compare lanes 3, 4 and 7 on figure 3A with lanes 3, 4 and 7 on figure 2A). Interestingly, II6/p44E166A and IIH6/XPDG675R mutated complexes, in spite of the absence of a direct strong interaction with p44, contained a substantial amount of XPD associated with the core complex (Figure 3A: lanes 1 and 5). The presence of XPD within TFIIH might be due to additional weak interactions with XPB and p62 (31, 33 and our unpublished data).

A run off transcription assay was carried out using the adenovirus major late promoter (MLP) as a template, the basal transcription factors, TBP, TFIIA, TFIIB, TFIIE, TFIIF and RNA pol II and as indicated, the various IIH6 complexes. The amounts of TFIIHs were adjusted on the basis of p62 and XPB content. First, the transcriptional activity of both IIH6/p44P100A (Figure 3B; lanes 13-14) and IIH6/p44R165A (lanes 17-18) was not affected by the mutations when compared to the recombinant wild type IIH6 (lanes 3-4). Second, transcription using either IIH6/p44G200R or IIH6/p44D66A was drastically diminished (Figures 3B lanes 7-8 and 15-16), reflecting on one hand the absence of interaction between XPD and the corresponding mutated p44 (Figure 2A: lanes 3 and 7) and on the other hand the weak association of XPD with the core complex (Figure 3A: lanes 3 and 7). The transcriptional activity of the IIH6/p44D178A complex did not exactly parallel the interaction between p44D178A and XPD and was lower than the expected level probably because of the low amount of XPD present in the IIH6 complex (Figure 3A: lanes 9-10). Additionally, the IIH6/p44E166A and IIH6/XPDG675R complexes were moderately active in spite of the presence of a normal amount of XPD in both complexes (Figure 3A: compare lanes 1 and 5 with lane 2).

CAK complex has been shown to stimulate the transcriptional activity when added to a reconstituted in vitro transcription assay (34). With the aim of knowing if the addition of CAK could circumvent any p44 defect, we carried out a run off transcription experiment in which CAK was added to the various IIH6 complexes (Figure 3C). p62 and XPB content were taken as a reference to adjust the amounts of TFIIH used in the run off transcription assay. We observed that addition of CAK does not modify qualitatively the ability of the mutated IIH6 complexes to
transcribe MLP. However, as previously observed the level of RNA synthesis was about 4-5 fold increased. This stimulation was even more pronounced when CAK is added to IIH6/p44E166A and IIH6/XPDG675R indicating that the CAK complex might stabilize XPD within TFIIH, through some interactions with other subunits of the core (35). Together these results show that mutations that affect the accurate p44/XPD interaction are detrimental for the transcriptional activity and that the effect of the CAK complex on transcription is mediated by this interaction.

Mutations in p44 lead to a defect in both the first phosphodiester bond synthesis and promoter escape:

It was recently demonstrated that the XPB helicase is responsible for the promoter opening and consequently facilitates the first phosphodiester bond formation (13, 20, 36) while XPD helps promoter clearance (22). Although XPD helicase activity seems to be dispensable for transcription initiation by RNA pol II, its role remains unclear, since clinical features of XP-D patients could not be explained only on the basis of NER defect (30). Nevertheless, XPD is absolutely required for CAK stimulation during transcription reaction likely because it anchors CAK to the core of TFIIH. The main question was to know whether the inhibition of transcription caused by mutations in p44 was due to an absence of stimulation of the XPD helicase activity and the impairment of CAK anchorage or to the inability of mutated p44 to fulfil a possible additional role in the transcription process.

We thus set up an abortive transcription initiation assay based on the detection of the synthesis of the first phosphodiester bond. Wild type IIH6 as well as mutated IIH6 were incubated with or without CAK in addition to the required general transcription factors, dinucleotides CpA, radiolabelled CTP as well as dATP as a source of energy and in the presence of MLP as a template. IIH6/p44P100A and IIH6/p44R165A showed a capacity to synthesize the first phosphodiester bond comparable to the wild type complex (Figure 4A, compare lanes 13-14 and 17-18 with lanes 3-4) as expected from the above transcription assay (Figure 3B and C, lanes 13-14 and 17-18). This observation also highlights the fact that the CAK complex enhances significantly the first phosphodiester bond synthesis level (Figure 4A, compare lanes 3, 13 and 17 with 4, 14 and 18 respectively). On the contrary, IIH6/p44G200R, IIH6/p44D178A, IIH6/p44D66A exhibited a very
strong deficiency in participating in the first phosphodiester bond formation (lanes 7-10 and 15-16). Both IIH6/XPDG675R and IIH6/p44E166A presented an abortive initiation defect, in spite of the presence of the XPD protein within the complex, meaning that an accurate interaction between p44 and XPD is required to allow the anchorage and/or the stimulatory effect of the CAK complex (compare lanes 6 and 12 with lane 4).

We were further interested in evaluating the ability of each mutant to clear the promoter after initiation. We carried out a promoter escape assay, independent of the role of TFIIH in promoter opening and abortive initiation (21). We therefore used a pre-melted heteroduplex DNA (-8/+2) as a template as described in Materials and Methods. To allow the accumulation of products that escape the promoter, we employed the chain terminating ATP-analog cordycepin. These products are mainly two oligomers of 17 and 31 nucleotides length. All recombinant TFIIH mutants that have a transcriptional defect also presented an impairment of promoter clearance except IIH6/p44P100A. IIH6/p44G200R, IIH6/p44D178A and IIH6/p44D66A showed a very strong inefficacy to perform the promoter escape (Figure 4B: compare lanes 7-10 and 15-16 with 3-4). This defect can be attributed to the absence of XPD within the three mentioned IIH6s (see figure 3A), or the consequence of p44 mutations. Moreover, CAK slightly stimulated the exit of RNA pol II from the promoter when added to IIH6wt, IIH6/p44P100A and IIH6/p44R165A (Figure 4B, compare lanes 3, 13 and 17 with lanes 4, 14 and 18 respectively) but significantly increased the escape when added to either IIH6/XPDG675R or IIH6/p44E166A (Figure 4B: compare lane 3 with 4 and lanes 5 and 11 with lanes 6 and 12 respectively). Nevertheless, the CAK complex increased the promoter clearance to a much lower extent than the first phosphodiester bond formation.

Together these observations highlighted the role of CAK in a very early step of transcription initiation reaction (for example compare figure 4A and B lanes 5-6, 13-14 and 17-18), whereas the physical presence of XPD (Figure 4B: compare lane 3 with 7, 9 and 15) and an accurate interaction between p44 and XPD (Figure 4B: compare lane 3 with lanes 5 and 11) seem to be of crucial importance for an efficient promoter escape reaction.
DISCUSSION

Transcription of protein coding genes is a multistep and complex mechanism which involves a battery of factors whose function is not yet fully elucidated. The understanding of gene expression became more difficult when transcription appeared to be coupled to DNA repair. In this aspect, TFIIH is a key actor because of its various enzymatic activities that are absolutely required for both mechanisms. One of the main difficulties to unravel the exact role of this factor is that TFIIH has to be considered as a whole complex whose subunits may be selectively involved in defined steps of transcription. For instance, we know that XPB helicase activity is crucial for promoter opening while XPD helicase activity is not. Nevertheless the physical presence of XPD is required for an optimal transcriptional activity (20, 22). In the present study we focused our attention on the role of p44, in particular its ability to stimulate the XPD helicase activity, and as a consequence its function in the first steps of the transcription reaction.

p44 regulates the XPD helicase activity.

It was previously shown in our laboratory that mutations in the C-terminal domain of XPD perturb its interaction with p44, thus explaining at least in part, the severe phenotypes observed in some XP-D patients (13, 24). To further investigate how such a contact was critical for the TFIIH activity, we undertook the analysis of the function of p44. Following a site directed mutagenesis procedure, we generated several point mutations in various conserved regions of the N-terminal part of p44. We found that mutations leading to amino acid changes at position 66 (D66E) and 200 (G200R) of p44 prevent its interaction with XPD and therefore do not allow an optimal XPD helicase activity (Table 1). Amino-acid changes at position 166 and 178 (E166A and D178A, respectively) weaken this interaction and partially inhibit the helicase activity of XPD, thus pointing out the clear correlation that exists between the XPD/p44 interaction and the XPD helicase activity. This study therefore enlightens the regulatory role of p44 towards the XPD helicase activity. In spite of the presence of all the signatures of an helicase protein, XPD works with p44, its regulatory subunit, to efficiently separate DNA strand. It thus appears clearly that the subsequent TFIIH
activities are tightly related to the nature of the interaction between XPD and p44 (Table 1). Indeed, the transcriptional activity of both IIH6/p44E166A and IIH6/XPDG675R complexes is weak and reflects the weakness of the interaction between the XPD and p44 subunits, in spite of the roughly normal TFIIH subunit stoichiometry. We can therefore assume that mutations of p44 that modify either its interaction with XPD or/and the proper architecture of TFIIH leads to the same defect as a mutation generated in the C-terminal domain of XPD (this study, see also 24). Moreover, this work delineates a p44 interacting domain in which amino-acids G200, D178, E166 and D66 are crucial for an accurate interaction with XPD. It is also worthwhile to point out that although p44 regulates the DNA unwinding activity of XPD, it has no effect on its ATP hydrolysis activity. In other words, the helicase activity and the ATPase activity of XPD are differently regulated.

The role of CAK in the transcription reaction:
The present work also sheds light on the CAK function. Indeed, the ability of CAK to stimulate the first phosphodiester bond formation depends on p44/XPD interaction. For example, we noticed that when p44 is mutated at position D178 or E166, the addition of CAK has a weak (if any) effect on the first phosphodiester bond reaction. In return, when p44 is mutated at position either P100 or P165, the stimulation by CAK is as high as for p44wt. In the absence of an accurate p44/XPD interaction the stimulatory effect of CAK is limited. It cannot be excluded that some p44 mutations diminish the efficiency of the first phosphodiester bond synthesis by affecting not only XPD binding but also the anchorage of the CAK complex to the core TFIIH. Indeed, in spite of the lack of interaction between p44 and XPD, this latter might still be part of the complex because of additional interactions of XPD with XPB but also p62 (31 and our unpublished results). Such interactions have also been reported in the yeast in which Rad3 protein binds directly to both SSL2/Rad25 and SSL1 proteins (33). Similarly, CAK binding to the core TFIIH, which is essentially mediated by XPD, might be stabilized through secondary interactions with other subunits of the core TFIIH such as XPB or p34 (35) to maintain the whole TFIIH complex in an
accurate conformation. We have noticed that some mutated IIH6 complexes which possess stoichiometric amounts of XPD exhibit a first phosphodiester bond synthesis activity much lower than the wild type complex, suggesting that this reaction requires not only the physical presence of XPD but also an accurate p44/XPD interaction.

Addition of CAK also increases promoter escape activity as a function of the interaction between p44 and XPD. Nevertheless, in the course of this study, we demonstrated that CAK has a much stronger stimulatory function during the first phosphodiester bond formation than during promoter escape. In the former case, the stimulation is up to 6-8 fold whereas in the later one, enhancement is much weaker. This suggest that the role of CAK occurs essentially in the first steps of the transcription: promoter opening and first phosphodiester bond formation, and is dependent of an accurate p44/XPD interaction.

Moreover, according to the above observations and regarding to the property of SSL1 in yeast, the role of p44 in human might not be restricted to transcription. Indeed, the G266R mutation in yeast (the counterpart of the G200R mutation in human) induces in addition to a transcriptional, a DNA repair defect, as well as a translational defect due to its incapacity to suppress the stem loops present in the mRNAs before translation (27). Furthermore, a T242I mutation in the yeast SSL1, is responsible for the stimulation of recombination between short repeats (<300 bp) (28, 40). This indicates that TFIIH may normally prevent these events, participating thus in the maintainance of the genome stability. Despite the pleiotropic effect of the modification of the p44 protein, these yeast mutants grow. Though SSL1 is an essential gene in yeast, nonlethal alleles, such as G266R, are temperature sensitive for growth, indicating a defect in protein function for TFIIH. Despite our efforts, no human patient bearing a mutation in the p44 gene, the human homolog of SSL1, has been reported so far. This might be explained by the fact that in human, the p44 gene is duplicated in the region q13 of chromosome 5 (25) and that both genes are expressed, thus making unlikely to find patients with XPD phenotypes. Nonetheless, this study demonstrates that mutations in the
human p44 gene, when tested in vitro, can cause a defect in TFIIH function, primarily resulting from defective assembly of the 9 subunit TFIIH complex.

It has to be pointed out that the contact domain which encompasses the C-terminal portion of Rad3/XPD as well as the N-terminal region of SSL1/p44 are highly conserved between yeast and human. This also strongly suggests that the XPD/Rad3 helicase regulatory function of p44/SSL1 was conserved during evolution and might, at least partially, explain the transcriptional defect of the SSL1 mutated yeast (26).

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Legends
Figure 1: Point mutations generated in the amino-terminal half of p44.

Point mutations have been introduced in the most conserved regions of the 5' half of the p44 cDNA by PCR. These mutations result in a conversion of an original residue into an alanin for five of the six mutations, D66->A, P100->A, R165->A, E166->A, D178->A and G200- R (the letters depict the considered residue and the numbers indicate the amino acid position). Position of mutations described in yeast is indicated in italic. The sequence alignements show the highly conserved amino acid through the evolution from yeast to human and plants (Hs stands for Homo sapiens, Dm for Drosophila melanogaster, Ce for Caenorhabditis elegans, Sp for Schizosaccharomyces pombe, Sc for Saccharomyces cerevisiae and At for Arabidopsis thaliana). A RING finger-like motif is present between residues 345 and 385, and a zinc finger between residues 291 and 308.

Figure 2: Interactions between p44 mutants and XPD and XPD helicase activity.
Lysates of Sf9 cells co-infected with wild type XPD baculovirus in addition to either of the p44 mutants baculovirus (Load) were immunoprecipitated (IP) with anti-XPD antibodies. The same procedure was followed with co-expressed wild type p44 with either wild type XPD (positive control) or G675R XPD mutant and with wild type XPD alone (negative control). After extensive washing of beads by a 0.4 M KCl buffer containing 0.1% NP40, half of the proteins remaining attached to the the beads were resolved on a SDS-PAGE followed by immunoblotting using antibodies directed towards p44 and XPD (panel A). The second half were tested in an helicase assay (panel B). (C): the same immunoprecipitated proteins as those described above were tested in an ATPase assay using in addition to proteins, (32P)γ-ATP as a substrate in the presence of DNA (lanes 3-10). The upper spots represent the released inorganic phosphate (Pi) whereas the lowest stands for non-hydrolysed substrate (ATP). Endogenous HeLa TFIH was used as a positive control (lane 2). The intrinsic instability of (32P)γ-ATP is represented on lane 1 and serves as a negative control. (D): increasing amounts of proteins were immunoprecipitated with anti-XPD antibodies coupled to protein G sepharose beads before incubation with (32P)γ-ATP in a regular ATPase assay.

Figure 3: Run off transcription assay with purified IIH6s and IIH9s.
(A): Immunopurified IIH6 complexes (IP) from baculovirus infected cell extracts (Load), as indicated at the top of the panels were submitted to SDS-PAGE and proteins were revealed by immunoblotting using antibodies raised against XPB, XPD, p62 and p44 subunits of TFIH. (B): Equal amounts of purified IIH6 complexes were tested in an in vitro transcription assay. HeLa TFIH has been used as a positive control while the assay run without any TFIH defines the
negative control. (C): Transcription performed as described above in the presence of fixed amount of CAK (2 µl). The size of the RNA run-off transcript is 309 nt.

Figure 4: Activity of wild type and mutated IIH6 in an abortive initiation and promoter escape assays: The immunopurified IIH6 complexes were tested for their ability to participate in the first phosphodiester bond formation (panel A; Abortive initiation) in the same conditions as a run off transcription but in the presence of CpA and \((^{32}\text{P})\gamma\text{-CTP, and dATP as a source of energy. The first phosphodiester bond formation is evaluated by the synthesis of a trinucleotide (3 nt) as indicated. Their ability to participate in the elongation process (panel B; Promoter escape) was measured using a premelted promoter template in the presence of ApG which served as an initiator and. Two major products with distinct lengths are synthesized as indicated.}

Table 1: summary of the different characteristics of each mutated TFIIH complex.
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Table 1

nd: non determined
p44/SSL1, the regulatory subunit of the XPD/RAD3 helicase plays a crucial role in the transcriptional activity of TFIIH

Thierry Seroz, Christophe Perez, Etienne Bergmann, John Bradsher and Jean-Marc Egly

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