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## Distinct Roles for the Helicases of TFIIH in Transcript Initiation and Promoter Escape\*

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**To provide an explanation of some clinical features observed within rare xeroderma pigmentosum (XP) patients and to further define the role of XPB, XPD, and cdk7, the three enzymatic subunits of TFIIH, in the transcription reaction, we have examined two defined enzymatic steps: phosphodiester bond formation and promoter escape. We provide evidence that the XPB helicase plays a dominant role in initiation, whereas the XPD helicase plays a minor contributing role in this step. The cyclin-activating kinase subcomplex of TFIIH improves the efficiency of initiation, but this involves only the structural contributions of cyclin-activating kinase rather than enzymatic activity. We demonstrate that XPB patient-derived mutants in TFIIH suffer from defects in initiation. Moreover, mutant analysis shows that in addition to its crucial role in initiation, the XPB helicase plays a critical enzymatic role in the promoter escape, whereas XPD plays an important structural role in the promoter escape process. Finally, using patient-derived mutations in TFIIH, we demonstrate deficiencies in promoter escape for both mutants of the class that suffer from combined xeroderma pigmentosum/Cockayne's syndrome.**

Transcription initiation of protein-coding genes involves a succession of events including the formation of a stable preinitiation complex, the ATP-dependent activation of the complex, promoter opening, first phosphodiester bond formation, and promoter escape. The fractionation of cell extracts from a number of sources has allowed the identification of a group of factors including TFIID, TFIIB, TFIIE, TFIIIF, and TFIIH, which in addition to RNA polymerase II are able to accurately transcribe several classes of promoters *in vitro*. Of all the polypeptides necessary to initiate the transcription reaction, enzymatic activities have been defined for only three: XPB, XPD, and cdk7, all of which are subunits of TFIIH (1–5). It is the recruitment of the multifunctional enzyme TFIIH to the promoter along with TFIIE and dATP that completes the preinitiation complex and commits a linear template to be transcribed (6). Some indication of the roles of these proteins has been provided by observations that negative supercoiling of

the template DNA relieves the requirements for TFIIH in basal transcription at multiple promoters (7–9). This has suggested that the TFIIH helicase activities are involved in open complex formation, first phosphodiester bond synthesis, and/or promoter escape, the requirements for which could be overcome by negative superhelicity in the template (7, 8, 10). Evidence of such roles for the helicases of TFIIH has been provided with the demonstration that this factor facilitates the melting of linear promoter DNA as shown by permanganate-footprinting experiments (11–14).

The identification of the two largest subunits of TFIIH showed, surprisingly, that these proteins are encoded by genes that are mutated in the rare autosomal recessive disorders xeroderma pigmentosum (XP).<sup>1</sup> Biochemical studies have thus demonstrated that the TFIIH is involved in both transcript initiation as well as DNA repair, the latter of which was initially considered to account for the phenotypic consequences of XP. However, the clinical complexity of this syndrome suggests that XP cannot be explained solely on the basis of NER deficiencies but may also involve transcription deficiencies. Previous studies have shown that mutations in the XPB helicase result in deficient opening of the adenovirus major late promoter (MLP) *in vitro* (13). One interest of these studies has been to determine if the enzymatic steps subsequent to promoter opening are deficient as well. In this study, we have investigated the roles of TFIIH and TFIIE in the initiation reaction. We provide evidence that they play a crucial role in promoter-specific, template-specified initiation on the adenovirus MLP *in vitro*. Moreover, we show that the XPB helicase of TFIIH, but not CAK, plays a critical enzymatic role in initiation. By contrast, the XPD helicase plays a minor, contributing role in initiation. Finally, we present evidence that the major role of CAK in transcription from MLP is to increase the efficiency of initiation, although not through any catalytic function of its own.

Beyond the roles in transcript initiation, the TFIIH helicases play a role in the subsequent step of promoter escape. This process involves the disruption of protein-protein interactions, which are necessary to initiate transcription as the RNA polymerase II moves beyond the promoter to adopt the elongation mode. Although previous studies have shown that TFIIH plays a critical role in this step, the contribution of each of the helicases has only recently emerged (7, 15, 16). In this report, we also have studied the roles of both XPB and XPD as well as CAK in the promoter escape reaction. Our data provide evidence that both helicases play a role in promoter escape. The XPB helicase plays an enzymatic role in the movement of the polymerase beyond this barrier to gene expression, whereas the

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<sup>1</sup> The abbreviations used are: XP, xeroderma pigmentosum; MLP, major late promoter; AdMLP, adenovirus MLP; nt, nucleotide(s); CS, Cockayne's syndrome CAK, cyclin-activating kinase; TTD, trichothiodystrophy; TBP, TATA-binding protein; NER, nucleotide excision repair.

XPD helicase activity appears to be dispensable, although its physical presence plays an important role in this step. This effect of XPD can be distinguished from the role of anchoring CAK to the TFIIF core; we provide evidence that the physical presence of CAK is dispensable to promoter escape at the AdMLP *in vitro*.

#### EXPERIMENTAL PROCEDURES

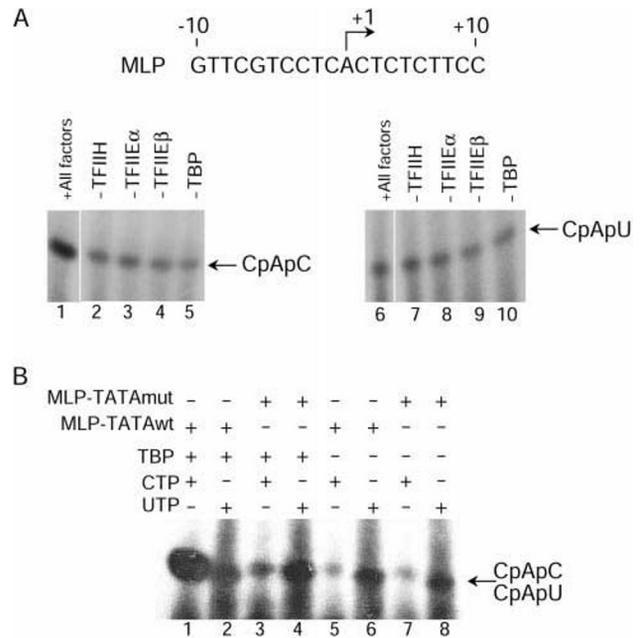
**Materials**—Ribonucleoside triphosphates and dinucleotides were obtained from Sigma. Cordycepin triphosphate (3'-deoxyadenosine triphosphate) was obtained from Sigma and TriLink (San Diego, CA).

**Transcription Factors**—Transcription reactions were performed using recombinant TFIIB, TFIIE, TFIIF, and TBP as well as endogenous TFIIF and RNA polymerase II as described (17). For recombinant TFIIF, two different purification schemes have been employed. Heparin Ultrogel (Sepracor, France) has been used as the initial purification step for each, although the objective of the purification (recombinant IIF6 core or IIF9 complex) determines the NaCl conditions for wash and elution of TFIIF as described followed by either an immunopurification of recombinant IIF9 complexes using antibody directed toward p44 (13) or by  $\text{Co}^{2+}$ -chelate affinity chromatography for His-p44-tagged IIF6 complex and the His-cyclin H-tagged CAK molecules (14).

**Abortive Synthesis Reactions**—Preinitiation complexes were assembled using 25 fmol of MLP template (pAK309 *EcoRI-SalI* fragment), 4 ng of TBP, 2 ng of TFIIB, 160 ng of TFIIF, 26 ng of TFIIE, and 200 ng of TFIIF (hydroxyapatite fraction) plus 1 unit of RNA polymerase II (HAP fraction) in a mixture containing 0.4 mg/ml bovine serum albumin and 5 mM  $\text{MgCl}_2$ . After preinitiation complex assembly at 28 °C for 30 min, phosphodiester bond synthesis was initiated by the addition of priming dinucleotides CpA (0.5 mM) plus  $\text{MgCl}_2$  at 6.5 mM, dATP at 4  $\mu\text{M}$ , and the appropriate radiolabeled nucleoside triphosphate at 1  $\mu\text{M}$  concentration. After 30 min of synthesis of the trinucleotide, the reactions were stopped in the presence of 100 mM EDTA and 0.5 mg/ml proteinase K. Routinely 10  $\mu\text{l}$  of such reactions were applied to a 20% acrylamide gel (19:1 acryl/bisacryl), 8.3 M urea gels and run at 20 W constantly for 2–2.5 h. In some reactions the runoff product was analyzed in parallel by the addition of CpA (0.5 mM),  $\text{Mg}^{2+}$  to 6.5 mM final concentration, dATP (4  $\mu\text{M}$ ), ATP, UTP, and GTP to 100  $\mu\text{M}$  and [ $\alpha$ - $^{32}\text{P}$ ]CTP to 1  $\mu\text{M}$  and cold CTP to 10  $\mu\text{M}$ . After 30 min such reaction products were extracted with phenol:chloroform, ethanol-precipitated, and run on 6–8% acrylamide, 8.3 M urea gels.

**Heteroduplex Templates**—For heteroduplex templates, an appropriate derivative of the AdMLP (–8/+2) region was generated, and both the derivative and the wild type AdMLP promoter plasmids (2 mg each) were then digested at unique sites on the opposite sides of the promoter region, using *HindIII* and *EcoRI*, respectively, in this case. For heteroduplex annealing, 400 mg of each plasmid was mixed with 40 ml of GM buffer (10 mM Tris-HCl, pH 7.8, 10 mM NaCl; 1 mM EDTA), then heated to 98 °C for 18 min and allowed to cool to room temperature slowly over 2 h, generating a population of heteroduplex and homoduplex molecules. After precipitation in ethanol, the DNA was resuspended and ligated for 30 s using 30 units of ligase in a final volume of 5 ml. The DNA was again precipitated with ethanol, resuspended and spun on a CsCl gradient to separate from nonligated, homoduplex DNA. Yield from this separation was 100–200  $\mu\text{g}$  of heteroduplex template. Template DNA was cleaved with *SalI*, phenol-extracted, and ethanol-precipitated to prepare for transcription. The template was designated MLP (–8/+2) heteroduplex.

**Promoter Escape Reactions**—Preinitiation complexes were assembled using 25 fmol of MLP (–8/+2) heteroduplex and 4 ng of TBP, 4 ng of TFIIB, 160 ng of TFIIF, 26 ng of TFIIE and 200 ng of TFIIF (HAP fraction), and 1–2 units of RNA polymerase II (HAP fraction) in a mixture containing 0.4 mg/ml bovine serum albumin and 5 mM  $\text{MgCl}_2$ . To initiate transcription in the absence of ATP, we used dinucleotide priming with ApG as the initiating substrate (see Fig. 3). After preinitiation complex assembly, transcription was started by the addition of 5 mM CTP and GTP, 1 mM radiolabeled UTP, 100 mM cordycepin, 4 mM dATP, and 6.5 mM  $\text{MgCl}_2$ . After 30 min at 28 °C, the reactions were stopped in the presence of 30 mM EDTA and 0.1 mg/ml proteinase K. Routinely 10  $\mu\text{l}$  of such reactions were applied to a 20% acrylamide gel (19:1 acryl/bisacryl), 8.3 M urea gels and run at 20 W constantly for 2–2.5 h. The generation of oligomers in this assay was completely ApG-dependent, arguing that ATP contamination is not sufficient to initiate transcription independently of the ApG dinucleotide. Finally, each of the major products beyond 14 nt in length was predicted by the initiation at –2 and termination at one of the downstream sites specifying adenosine in the growing RNA chain, ruling out spurious tran-



**FIG. 1. Trinucleotide synthesis observed in the absence of TFIIE and TFIIF can be accounted for as template sequence-nonspecific synthesis.** *A*, the dinucleotide CpA was used to prime trinucleotide synthesis from the MLP template in the presence or absence of TFIIF, TFIIE $\alpha$ , TFIIE $\beta$ , or TBP as indicated at the top of each panel, in the presence of radiolabeled CTP or UTP to detect the template nonspecific component. *B*, abortive trinucleotide synthesis from either MLP-TATAmut or MLP-TATAwt (at 25 fmol/reaction) carrying a 3-point mutation in the TATA-box (19) was used as a template using CpA dinucleotide and radiolabeled NTP in the presence or absence of TBP and all the other transcription factors as indicated at the top of the figure.

scription as the origin of our oligonucleotides. To provide confirmation of the exact length of the oligonucleotides synthesized, parallel reactions were performed in which templates contained G-less cassettes downstream of the AdMLP. Oligonucleotide synthesis in the presence of ATP, UTP, and CTP thus allowed a direct comparison of the promoter escape products with oligomers of similar length.

**Cell Strains and Culture Conditions**—Cell lines HD2 (derived from XP102LO) and HeLa were grown in Dulbecco's medium supplemented with 10% fetal calf serum and antibiotics.

#### RESULTS

Previous work from our laboratory strongly suggested that mutations found in XP patients affect the early steps in transcription. To provide an explanation of some clinical features observed within the rare XP patients and to further define the role of XPB and XPD in the transcription reaction, we have examined two defined enzymatic steps and have characterized these pathogenic mutations with respect to their deficiencies in each.

**Promoter-specific Initiation Requires the XPB Helicase Activity of TFIIF**—Previous work from several laboratories including ours suggested a role for TFIIF in promoter opening (11, 13, 14, 18). We have thus evaluated the requirement of TFIIF and TFIIE for synthesis of the first phosphodiester bond using the abortive initiation reaction, in which a priming dinucleotide replaces the NTP specified at the predominant start site of the adenovirus MLP. Such initiation from the MLP in the presence of all the basal transcription factors including TFIIF and RNA polymerase II generated trinucleotides (CpApC) that have a cytidylyl extension on the 5' end (Fig. 1A, lane 1). By providing the dinucleotide ApC and UTP, one can similarly monitor the synthesis of the first phosphodiester bond specified by initiation at the +2 site of the MLP, generating ApCpU (data not shown). Both reactions were reconstituted *in vitro*

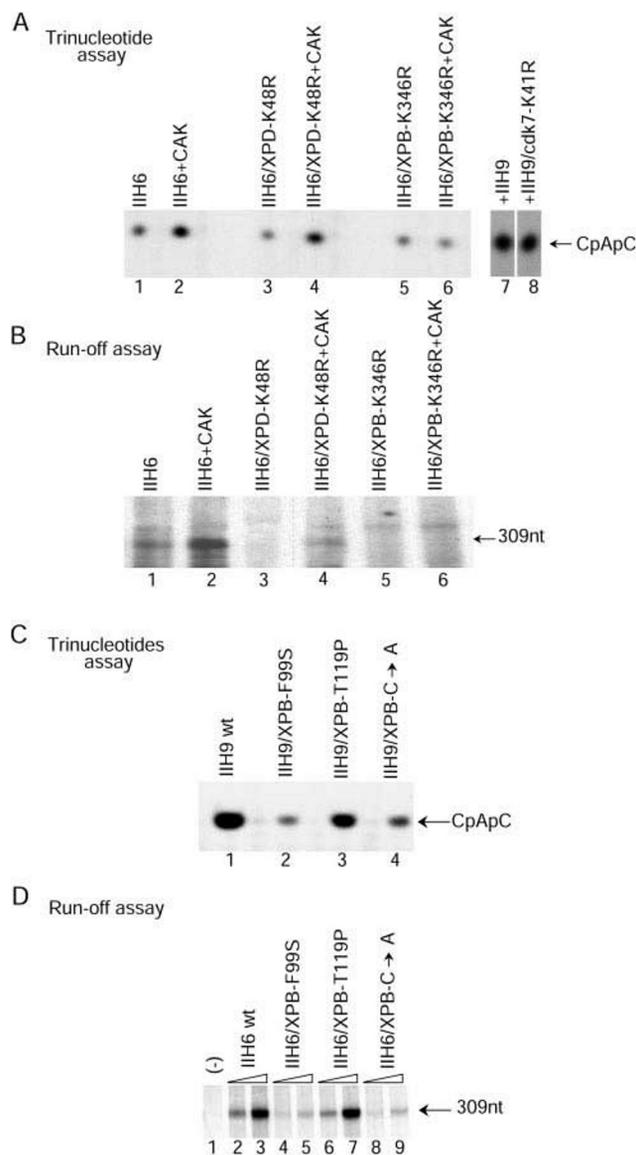
according results from S1 nuclease mapping of the start sites of the MLP (data not shown). The synthesis of the MLP accurate product, CpApC, also occurs in the absence of TFIIH as well as either subunit of TFIIE (Fig. 1A, lanes 2–4). Similarly the synthesis of CpApU, inaccurate with respect to MLP, also occurred independently of the presence of TFIIH and TFIIE (Fig. 1A, lanes 7–9). However, in both cases, the synthesis of the trinucleotide is comparable with that from reactions lacking TBP, suggesting that such quantities of trinucleotide are synthesized promoter nonspecifically (Fig. 1A, lanes 5 and 10). Studies of the fate of these template sequence-nonspecific (CpApU) initiation products have shown that elongation only occurs if the initiation product is specific to the template sequence (data not shown), again supporting the view that TFIIH is crucial to the initiation of productive transcription.

We have also evaluated the promoter specificity of initiation by looking at the dependence on the TBP-TATA box interaction, which nucleates the formation of the preinitiation complex. Synthesis of either the template-specific product CpApC or the template-nonspecific product CpApU has been determined in separate reactions either in the absence of TBP or in the presence of a down-mutated MLP (a 3-nt mutation destroying partially the TATA-box, designated MLP-TATAmut) (19). Optimal trinucleotide synthesis only occurs in a template-specific manner when MLP-TATA wt and TBP are present (Fig. 1B). These observations suggest that a fraction of the total synthesis is not specified by the formation of the preinitiation complex, a phenomenon we refer to as promoter-nonspecific initiation. Also, these data show that even when the components necessary to establish promoter-specific initiation are present (*i.e.* promoter sequence and all basal factors), initiation can occur in a template sequence-nonspecific fashion. As previously mentioned, only the template sequence-specific initiation products are chased into higher molecular RNA (data not shown). Finally, these results agree with previous studies that have concluded that optimal initiation occurs in the presence of all transcription factors including TFIIH (7, 20, 21).

**Distinct Role of XPB and XPD Helicases in Transcription—**Using a baculovirus expression system to generate recombinant wild type and mutant versions of TFIIH complex (including the entire TFIIH, IIH9; the core-TFIIH/XPD, IIH6; and the cyclin-activating kinase, CAK, which contains the cyclin-dependent kinase cdk7 in addition to cyclin H and MAT1) (22–24), we found distinct functions for each helicase of TFIIH. Indeed, the complete TFIIH complex (IIH6/XPB-K346R + CAK) (25) containing an ATP binding site mutation in the XPB helicase (26) was deficient in the synthesis of the first phosphodiester bond (Fig. 2A, compare lanes 2 and 6). In contrast, the complete TFIIH complex with a similar mutation to the XPD helicase (IIH6/XPD-K48R + CAK) retained a significant fraction of the initiation activity seen with the wild type complex (lanes 2 and 4), corroborating previous results (16).

A more pronounced difference between the wild type and IIH6/XPD-K48R was seen in the synthesis of full-length transcripts, in which only 10–20% of the wild type protein activity was observed with the XPD mutant (Fig. 2B, compare lanes 2 and 4). These results strongly suggested that the XPD helicase plays a role in transcription subsequent to the initiation step. We have also observed that reactions with IIH6/XPB-K346R results in a complete absence of runoff transcripts (lanes 5 and 6), consistent with the interpretation of a deficiency in promoter opening and initiation with this protein (Ref. 14 and Fig. 2A).

The abortive synthesis assay also allows us to analyze the influence of CAK on the initiation reaction. When CAK was added to either IIH6 wild type or to IIH6/XPD-K48R, we observed a significant stimulation of the trinucleotide synthesis



**FIG. 2. A crucial role for XPB and CAK during the first phosphodiester bond formation.** A and B, trinucleotide (abortive) and runoff assays were performed in the presence of all the basal transcription factors and either recombinant IIH6, IIH6/XPD-K48R, or IIH6/XPB-K346R mutated in their consensus ATP binding sites, in the presence or absence of CAK as indicated at the top of the figure. C and D, abortive and runoff assays were performed similarly for the patient-derived mutations IIH9/XPB-F99S, IIH9/XPB-T119P, or IIH9/XPB-C/A, all in the presence of the ternary CAK complex. The CpApC as well as the 309-nt runoff products are indicated by an arrow.

(Fig. 2A, compare lanes 1–2 and 3–4). In contrast, reactions performed with IIH6/XPB-K346R were not stimulated in the presence of CAK (Fig. 2A, lanes 5 and 6), thus illustrating the dependence of CAK stimulation on the functional XPB helicase. To investigate the dependence of the initiation reaction on the cdk7 kinase activity, we tested the activity of IIH9/cdk7-K41R, in which cdk7 was mutated in its ATP binding site and, thus, no longer has any kinase activity (27). We found that TFIIH complex containing the mutant cdk7 was as efficient as a complex with wild type cdk7 in forming the initial phosphodiester bond (Fig. 2A, lanes 7 and 8). Together, these results argue that the major effect of the ternary CAK complex in transcription from CAK-independent promoters such as MLP (14, 28) is to improve the efficiency of the initiation reaction, although not through any catalytic effect of its own. Furthermore, this is strictly dependent on the activity of the

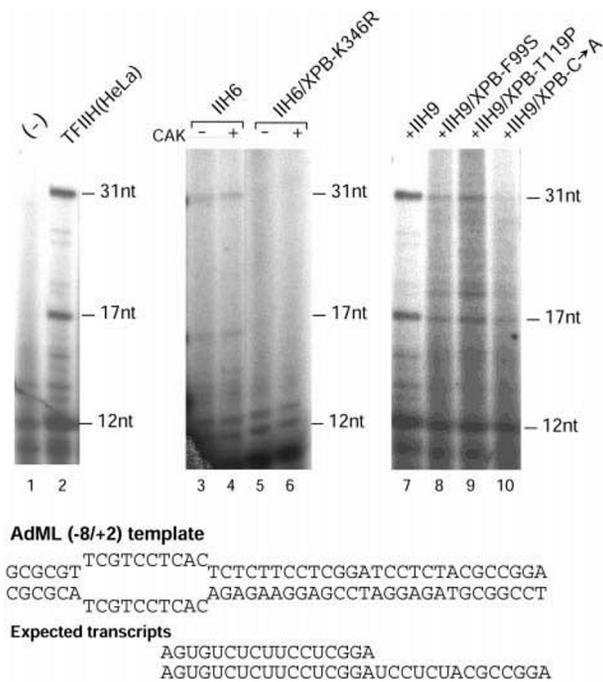
XPB helicase.

**Mutations Found in XPB Patients Affect the Early Steps of Transcript Initiation**—Continuing an analysis of the role of XPB in the transcription reaction, we have checked the activity of XPB pathogenic mutations found in three rare XPB patients (Refs. 29, 30, and 38).<sup>2</sup> Two of these mutations, F99S and C/A, cause a combined XP/Cockayne's syndrome (CS) phenotype due to a point mutation (F99S) or to a transversion that resulted in a splice mutation and a shifted reading frame for the C-terminal 40 residues of XPB (C/A). The remaining mutant, T119P, caused a combined XP/trichothiodystrophy (TTD) phenotype. These three XPB mutations have been introduced into recombinant expression constructs, and the resulting baculoviruses were used for coexpression with the other eight subunits of TFIIH. The abortive initiation assay using the immunopurified IIH9 complexes (13) gives a clear indication that the F99S and C/A mutants are somewhat deficient in the formation of the first phosphodiester bond, whereas the T119P mutant retains a significant fraction of the activity of the wild type protein (Fig. 2C). Similar deficiencies were also observed in the assays for runoff transcription (Fig. 2D). In conclusion, these results provide evidence that two of these pathogenic mutations have some deficiencies in transcript initiation (first phosphodiester bond formation), which are expected to play a dominant role in the expression of genetic information *in vivo*.

**Patient-derived XPB Mutations Affect Promoter Escape**—Our analysis has also addressed promoter escape for the XPB mutations found in the patients. To make these assays independent of the roles of TFIIH in initiation, we employ a pre-melted heteroduplex DNA as the template. Use of the chain-terminating ATP-analog cordycepin allowed the accumulation of products that escape the promoter as a group of oligomers of 17- and 31-nt length, whereas shorter oligos (12 nt), indicative of polymerases that fail to clear the promoter, are observed in the absence of TFIIH. In these experimental conditions, we have observed that the XPB mutant (IIH9/XPB-K346R) did not allow RNA polymerase II to escape the promoter (Fig. 3A, compare lanes 1–2 and 3–4). CAK played no apparent role in these reactions; the ability of both the wild type and IIH6/XPB-K346R to escape the promoter was unchanged in its presence (Fig. 3A). This observation supports the idea that the primary effect of CAK on CAK-independent promoters such as MLP is to increase the efficiency of the initiation reaction (Fig. 2).

Patient-derived mutations to XPB do appear to have a strong effect on promoter clearance. Compared with the wild type IIH9 reaction in which the 17- and 31-nt oligomers were generated, the synthesis of cordycepin-stop oligomers of lengths 17 nt and greater appears reduced with either the IIH9/XPB-F99S or the IIH9/XPB-C/A mutant proteins (Fig. 3, lanes 8 and 10 compared with lane 7). This suggests that pathogenic mutations for combined XP/CS (F99S and C/A) suffer from similar deficiencies in the promoter escape process. One other mutation causative of combined XP/TTD, the T119P mutation, appears to have only a modest deficiency in promoter escape, generating near wild type levels of the 17-nt and 31-nt oligomers (Fig. 3, lanes 7 and 9). We have independently confirmed these results in assays using the immunoprecipitated protein from those cell lines established from affected patients (data not shown). In conclusion, we have defined a promoter escape deficiency for two pathogenic mutations to TFIIH, both of which also have initiation deficiencies and thus appear to have compound defects in expression of genetic information.

**XPB Plays a Role in Promoter Escape**—The above results suggested a predominant role of XPB in some post-initiation



**FIG. 3. Promoter escape requires active XPB.** Transcription employed a pre-melted heteroduplex DNA as the template, which is single-stranded between nt -8 and +2, as indicated at the bottom of the panel. To initiate transcription efficiently, we used the dinucleotide ApG in a 40-fold excess over the concentration of GTP in the reactions. We interpreted the heterogeneity of the group of cordycepin-stop oligomers as an indication of ATP contamination in the assay, allowing for read-through of the promoter-proximal stop sites (17 nt) to generate the more promoter-distal of the oligomers (31 nt). However, regardless of the prospect of ATP contamination, these promoter escape oligonucleotides are initiated uniformly. Each of the major products beyond 14 nt in length was predicted by the initiation at -2 and termination by a downstream site specifying adenosine in the growing RNA chain. Promoter escape reactions using equivalent amounts (according to Western blot) of immunopurified IIH from HeLa cells (lane 2) or a recombinant source of either TFIIH or CAK (lanes 3–10) were prepared as described under "Experimental Procedures."

events (Fig. 2). We have previously found that IIH6/XPB-K48R was able to support promoter escape with efficiency similar to the wild type (data not shown). Considering that the helicase activity of XPD is dispensable to promoter escape, we wondered if the presence of the XPD subunit made essential contributions to the escape reaction. To address this, TFIIH was purified from HD2 cells, which bear a mutation to XPD (IIH-XPDR683W). HD2, a cell line that is derived from the XP102LO patient, carries a R683W mutation in the C-terminal end of XPD in one of the alleles and an L463V change as well as a 716–730-amino acid deletion in the other one (31). However, a recent study indicated that L463V 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 (32). Immunopurification of TFIIH results in a complex with an altered association between XPD and p44; consequently, the TFIIH contains principally the 5 core subunits XPB, p62, p52, p44, and p32, as observed by Western blotting (Fig. 4, left panel). We have previously shown that the weakened association between XPD and p44 disrupts the stimulatory influence of p44 on XPD, thus diminishing the XPD helicase activity of TFIIH and causing a deficiency in transcription (13).

The transcription complexes assembled with such TFIIH are not proficient to fully escape the promoter (compare Fig. 4, right panel, lane 1 with Fig. 3, lane 1). The addition to these reactions of the partially purified HeLa cell CAK/XPB (Fig. 4, left panel) was able to qualitatively restore the promoter escape

<sup>2</sup> A. Sarasin, unpublished results.

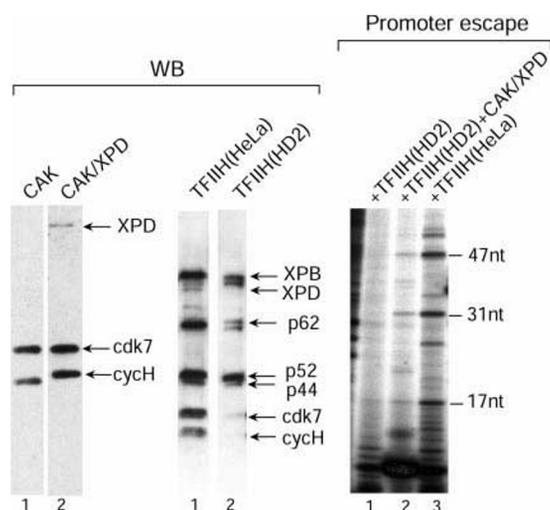


FIG. 4. A role for XPD in promoter escape that can be distinguished from anchoring CAK to the core TFIIH. Promoter escape reactions (right panel, lanes 1 and 2) were performed as described using immunopurified TFIIH from either HeLa or HD2 cells (see Western blot (WB), middle panel). To circumvent the effect of XPD mutation, conventionally purified CAK/XPD protein (left panel) was added in the reaction (lane 2).

pattern to that of wild type TFIIH immunopurified from HeLa cells (Fig. 4, right panel, compare lanes 1 and 2 with lane 3), generating oligonucleotides of 17 and 31 nt as predicted for termination with the ATP analog cordycepin. Our previous studies have shown that CAK plays no role in the promoter escape from the AdMLP, indicating that the effect of XPD can be distinguished from anchoring CAK to the core TFIIH (Fig. 3). In conclusion, we have shown that promoter escape requires the physical presence of XPD and that this requirement can be distinguished from the role of XPD in anchoring CAK to the core TFIIH (see Fig. 4).

#### DISCUSSION

In an effort to understand the mechanisms that underlie the expression of protein-coding genes, we have investigated the role of TFIIH in the first steps of the transcription reaction. This study, which was made possible with designed recombinant TFIIH complexes and immunopurified TFIIH from either HeLa cells or XP patient-derived cell lines, intends to describe the transcriptional defects of XP mutations. We first demonstrated that template sequence-specific initiation, *i.e.* first phosphodiester bond formation, absolutely requires TFIIH as well as TFIIIE. Our data however indicate also that in addition to the promoter-specific component, the abortive synthesis assay is able to generate a significant promoter nonspecific component independently of the formation of the stable preinitiation complex initiated by the TATA box-TBP interaction.

**XPB Is Crucial for Formation of the First Phosphodiester Bond**—We provide direct evidence that the XPB helicase, one of the core-TFIIH subunits is shown to play a direct role in initiation; mutations to the consensus ATP binding motif of XPB reduce transcript initiation to that seen in the absence of a preinitiation complex (the present study, see also Ref. 16). Previous studies have shown that the ATP binding site mutation designed in XPB does not promote the opening of the promoter around the start site, an essential step that allows RNA polymerase II to initiate RNA synthesis (14). Moreover, there is a strong correlation between the degree of opening of the promoter as detected by permanganate footprinting and the level of RNA synthesis (13). Additional evidence supporting this correlation between formation of an open promoter structure and initial phosphodiester bond synthesis comes from our

studies of the patient-derived mutations to XPB. We have previously observed a serious deficiency in forming an open promoter for the XPB C/A mutant and a partial deficiency for the XPB/F99S mutant (13). Currently it is unknown what degree of opening of MLP is essential to promote synthesis of the initial phosphodiester bond. Our present data show that, despite a significant difference in the promoter opening activities of these mutants, a similar deficiency in initiation is observed for both, suggesting that a rather complete opening at MLP may be necessary to stimulate initial phosphodiester bond synthesis. Further studies may allow us to draw a closer correlation between promoter opening, initial phosphodiester bond synthesis, and their dependence on XPB.

**Pathogenic XPB Mutations Have Distinct Deficiencies in the Early Stages of Transcription**—First, we have provided evidence that the helicase of XPB plays a crucial role in promoter escape. Consistent with the crucial role of XPB activity in this process, we show that some naturally occurring mutations to XPB (XPB/CS) have deficiencies in the steps of both transcript initiation and promoter escape. By contrast, one mutation found in combined XPB/TTD demonstrated suboptimal initiation but near normal promoter escape. These observations are consistent with the deficiencies of these proteins in assays for promoter opening (13) and again argue that the XPB subunit is necessary not only for promoter opening but also for formation of the first phosphodiester bond from a linear MLP *in vitro*. *In vivo* the XPB deficiency in initiation is expected to play a dominant role in the suboptimal expression from promoters like MLP; however, it is clear that initiation occurs on negatively supercoiled templates independently of TFIIH (8). Template sequence may play a role in the dependence of initiation on TFIIH as well. In the case of such templates, the dominant effect of XPB deficiencies in transcription may be observed during promoter escape or subsequent, uncharacterized steps of gene expression that are dependent on TFIIH. Such promoter sequence and topological conformation effects may thus strongly influence the deficiencies of these mutants observed in a broader class of promoters tested.

These data also provide an insightful distinction for mutants in the same protein (XPB), which cause dramatically different clinical phenotypes of combined XP/CS or combined XP/TTD. Although it seems difficult to rationalize how mutations to the same protein give dramatically different clinical pictures, it is of some benefit to know that the combined XP/CS disorder appears to have a compound deficiency in transcription. In this group of mutants, both transcript initiation and promoter escape are suboptimal. By contrast, in the sole representative of the combined XP/TTD, a rather simpler picture emerges. The mutant proteins appear to suffer a deficiency in initiation, but promoter escape appears near normal. In the latter case the single, mildly affected patient has only been shown to have defects in NER.<sup>3</sup> although a more comprehensive study of the deficiencies in a broader class of mutants of each group is in order, these observations provide a framework for understanding the deficiencies of expression of genetic information in these two clinical syndromes.

**The XPD Protein Plays a Role in Promoter Escape**—The subordinate role of XPD in transcription is supported by the frequency of patients that suffer from defects in XPD as compared with those that suffer from XPB defects. Previous work also shows that the helicase activity of XPD is not essential in transcription (14, 16). Here we have shown that XPD plays a role in the promoter escape reaction, although the ATPase/helicase activities of XPD appear to play no role in this process.

<sup>3</sup> A. Sarasin, personal communication.

We find that this data provides a strong parallel and possible explanation for the requirement of RAD3 in transcription in yeast. RAD3 has a DNA-dependent ATPase and DNA helicase activity and was identified as the yeast homolog of XPD (33). Although essential, RAD3 has nonlethal alleles, one of which, rad3-21, substitutes a glutamic acid for a lysine residue in the nucleotide binding domain. Although cells bearing this mutation have an increased sensitivity to UV radiation, transcriptional activity is normal (34, 35). These data suggest that 1) the essential requirement for RAD3 may not be related to its involvement in NER but may instead reflect its involvement in transcription, and 2) the transcriptional effect of RAD3 may not require its ATPase/DNA helicase activity. Our observations that the helicase activity of XPD is dispensable to transcription, although the presence of the protein plays an essential role to promoter escape, provide a functional link to the mammalian system. We have shown that the first two enzymatic steps of RNA synthesis, encompassing initiation and escape of the polymerase to +31, do not absolutely require the ATPase/DNA helicase activity of XPD; however, promoter escape appears strongly dependent on the physical presence of XPD. The evidence that CAK is dispensable to the process of promoter escape supports the idea that the stimulatory effect of XPD can be distinguished from the ability to anchor CAK to the core TFIIH. These results also help to explain our previous observation that the addition of IIH5 to a pre-melted promoter was unable to completely restore the level of transcription (13). These studies show that the addition of wild-type XPD in the CAK-XPD complex to IIH5 is necessary to restore promoter escape.

Although the equilibrium between core TFIIH including XPD and CAK, two subcomplexes of TFIIH (25, 36, 37), plays an important role in RNA synthesis, these studies have discovered another important equilibrium within the TFIIH complex. As shown in Fig. 2, the addition of CAK is able to restore the efficiency of initiation when added to the IIH6 core (including XPD). Similar additions of CAK in the promoter escape reactions provided evidence that the IIH6-CAK equilibrium plays no role in this process. However, IIH5 from HD2 cells, which bear a mutation to XPD that destabilizes the interaction between XPD and p44, was able to qualitatively restore the promoter escape pattern to that of the wild type TFIIH (Fig. 4). This result provides evidence that the equilibrium between XPD and the five-subunit core TFIIH found in HD2 cells can play an important role in promoter escape.

*The Physical Presence of CAK, although Not Its Enzymatic Function, Stimulates Initiation*—We also have shown that the ternary CAK complex plays a strong stimulatory role in formation of the first phosphodiester bond; however, the enzymatic activity of cdk7 plays no role in the efficiency of transcript initiation in this system. This is consistent with the observation that CAK activity is dispensable to transcription from the AdML promoter *in vitro* (14, 28). Indeed, we have found that CAK with catalytically inactive cdk7, when used for the initiation reaction, has the same effect as the wild type counterpart. These observations support the interpretation that the effect of CAK upon initiation from the AdML promoter *in vitro* is to increase the efficiency of the initiation reaction without catalytic involvement of its own. This may likely occur as a result of improving the structural stability of the preinitiation complex. Our previous observations have shown the interaction of CAK with the XPB subunit of TFIIH (25), suggesting that CAK may improve the functional interaction of XPB with the template DNA to promote helical unwinding at the transcriptional start site.

In summary, upon association of TFIIH to complete the

preinitiation complex, a promoter is able to be active in transcription. The initial enzymatic steps that allow productive transcription include the helical unwinding of the template and the formation of the initial phosphodiester bond, both of which rely on the XPB helicase. These studies have also shown that the ternary CAK complex stimulates the efficiency of initiation, likely through stabilization of the preinitiation complex. After the formation of 10–14 phosphodiester bonds, the polymerase encounters a second barrier to gene expression: promoter escape. In this step, the XPB helicase again plays a fundamental role in progress past this barrier; however, in contrast to initiation, this step occurs independently of CAK. Although the activity of the protein is dispensable, XPD also plays an important role in this step, possibly by stabilizing the five-subunit core TFIIH.

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