Novel Functional Interactions between Nucleotide Excision DNA Repair Proteins Influencing the Enzymatic Activities of TFIIH, XPG, and ERCC1-XPF†

G. Sebastiaan Winkler,‡ Kaoru Sugasawa,§ André P. M. Eker, Wouter L. de Laat, and Jan H. J. Hoeijmakers*

Department of Cell Biology and Genetics, Medical Genetics Center, Center of Biomedical Genetics, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

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

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

Six human protein factors are required for recognition and dual incision in vitro (*5*, *6*). The XPC-HR23B complex binds with high specificity to DNA damage and represents the initial recognition factor able to recruit the remainder of the NER apparatus (*7*). Three additional factors are required for the subsequent ATP-dependent local melting of the DNA helix around the lesion (*8*−*10*): basal transcription factor IIH (TFIIH), which has an additional, essential cellular function in transcription by RNA polymerase II (see refs *11* and *12* for further details); XPA, which preferentially binds to DNA containing lesions as well as single-stranded DNA (*13*); and the single-stranded DNA binding protein RPA, which most likely binds to the undamaged strand (*14*). The locally unwound region provides a substrate for the structure-specific endonucleases XPG and ERCC1-XPF, which incise the damaged strand at the 3′ and 5′ side, respectively (*15*, *16*).

TFIIH possesses several enzymatic functions: the DNA-dependent ATPase and DNA helicase activities are attributed to both of the largest subunits, XPB and XPD (*17*, *18*). CDK7 was identified as the kinase catalytic subunit, which is able to phosphorylate the C-terminal domain of the largest subunit of RNA polymerase II during initiation of transcription (*19*, *20*). In NER, TFIIH has also been implicated with a function in DNA unwinding by virtue of its helicase subunits (*9*, *21*). To obtain further mechanistic insight into the role of TFIIH and ATP during the dual incision step, we have analyzed how the enzymatic activities of TFIIH are influenced by the other five NER incision factors and vice versa and identified a novel function for TFIIH and ATP distinct from DNA unwinding.

MATERIALS AND METHODS

Purification of NER Factors. Recombinant mouse XPA and human RPA were both purified from *Escherichia coli* strain BL21(DE3)pLysS using plasmids pET-8c-XPA (kindly provided by Drs. C. F. van Kreyl and H. van Steeg) and p11d-tRPA (a generous gift of Dr. M. S. Wold) exactly as described previously (*7*, *22*). Human XPC-HR23B complex

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* Corresponding author. Telephone: +31 10 408 7199. Fax: +31 10 408 9468. E-mail: hoeijmakers@gen.fgg.eur.nl.

‡ Present address: Imperial Cancer Research Fund, Clare Hall Laboratories, Blanche Lane, South Mimms, EN6 3LD, U.K.

§ Present address: Cellular Physiology Laboratory, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-01, Japan.

[1] Abbreviations: NER, nucleotide excision repair; UV, ultraviolet; XP, xeroderma pigmentosum; TFIIH, transcription factor IIH; AAF, acetylaminofluorene.

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was reconstituted from recombinant XPC, purified from baculovirus-infected insect cells, and HR23B purified from *E. coli*, as described (*23*, *24*). Human XPG endonuclease was obtained from insect cells infected with recombinant baculovirus (generously provided by Dr. R. D. Wood) using a procedure described before (*14*). Recombinant human ERCC1-XPF complex was partially purified from *E. coli* according to a procedure that will be described in detail elsewhere. Highly purified TFIIH was obtained either from human fibroblasts expressing epitope-tagged XPB (*25*) or from HeLa cells using classical chromatography (*26*). TFIIH containing fractions from the last steps of either purification were used and gave identical results.

Analysis of ATPase Activities. Stimulation of the TFIIH-associated ATPase activity was analyzed in 10-μL reaction mixtures (*25*, *27*). Reaction mixtures contained approximately 100 ng of TFIIH, 50 ng of XPA or XPC-HR23B complex, and 100 ng of DNA as indicated. Reactions were terminated by the addition of 5 μL of 0.5 M EDTA, and 0.2 μL of each reaction was analyzed by thin-layer chromatography on polyethylenimine−cellulose plates (Merck) (*25*).

Analysis of Nuclease Activity. Nuclease reactions were carried out in 15-μL volumes in buffer containing 0.75 mM $MnCl_2$ (*14*). As a model substrate, a hairpin of 22 bp with either a 3′-end or 5′-end protruding-d$(T)_{28}$ arm was used as described previously (*14*). Per reaction, 125 ng of purified XPG endonuclease or approximately 1.0 μg of protein fraction of partially purified recombinant ERCC1-XPF complex was added to 1.5 ng of labeled substrate. Inhibition by TFIIH (approximately 100 ng) was reversed in the presence of 1 mM ATP. Availability of the restriction enzyme site in the duplex region was analyzed by addition of *Hae*III (10 units; Boeringer Mannheim) to a pre-assembled reaction mixture.

RESULTS

Stimulation of TFIIH-Associated ATPase Activity by XPA and XPC-HR23B. Hydrolysis of the $\beta-\gamma$ bond of ATP by TFIIH is an essential step during NER, and at least part of the energy is required for the formation of a melted region around DNA damage (*8*, *10*). In addition to TFIIH, three protein factors are minimally required for local DNA unwinding: XPA, XPC-HR23B, and RPA (*8*−*10*), which may stimulate the DNA-dependent ATPase activity of TFIIH. To find evidence for this hypothesis and identify individual factors involved, the effect of these proteins on the ATPase activity of TFIIH was analyzed using purified proteins (Figure 1A). In the absence of DNA, the levels of TFIIH-dependent ATP hydrolysis were <2% for all protein preparation tested (data not shown; for XPA, RPA, and XPC-HR23B see also levels of ATP hydrolysis in the presence of DNA, Figure 1B). As reported (*25*), ATP hydrolysis by TFIIH was stimulated by DNA but was not further stimulated by the presence of AAF damage in the DNA (compare both panels in Figure 1B). However, while purified XPA alone did not have any influence on ATP hydrolysis, the TFIIH-associated ATPase activity was strongly increased in the presence of both XPA and damaged DNA (Figure 1B). This effect was clearly greater than additive and thus indicates a stimulatory role of XPA on ATP hydrolysis by TFIIH. Furthermore, this effect was specific for the presence of DNA damage because undamaged DNA failed to stimulate the TFIIH-associated ATPase in the presence of XPA. Interestingly, purified XPC-HR23B complex, which alone did not hydrolyze ATP, was also able to stimulate the TFIIH-associated ATPase (Figure 1B). However, although the stimulation by XPC-HR23B was observed only in the presence of DNA, no additional specificity for DNA lesions was present. The stimulatory effects of XPA and XPC-HR23B on the TFIIH-associated ATPase were specific, as RPA did not not stimulate the hydrolysis of ATP by TFIIH in either the absence or the presence of (damaged) DNA (Figure 1B).

When RPA was added to reactions containing both XPA and TFIIH or XPC-HR23B and TFIIH, only little effects on ATP hydrolysis were observed as compared to reactions carried out in the absence of RPA. However, whereas RPA appeared to have a marginal positive effect on ATP hydrolysis in the presence of XPA and TFIIH, it appeared to give rise to a slight inhibition of ATP hydrolysis in the presence of XPC-HR23B and TFIIH (Figure 1C). In reactions containing XPA, XPC-HR23B, and TFIIH, stimulation of ATP hydrolysis was detected in the presence of both damaged and undamaged DNA (Figure 1C). No synenergistic stimulation was observed, which indicated that the interaction between XPC-HR23B and TFIIH was preferred under the conditions used.

To further substantiate the specificity of the stimulatory roles of XPA and XPC-HR23B on ATP hydrolysis by TFIIH, we investigated the effect of these proteins on the ATPase activity of a heterologous NER factor, the *E. coli* UvrA protein. Both XPA and XPC-HR23B did not stimulate the ATPase activity of UvrA (Figure 1D). By contrast, UvrB did stimulate the ATPase activity of UvrA specifically in the presence of damaged DNA, indicating that the UvrA protein used is responsive to a specific interaction (Figure 1D). These data thus provide further evidence for the specificity of the stimulation of the TFIIH-associated ATPase activity by XPA and XPC-HR23B.

ATP-Reversible Inhibition by TFIIH of the XPG Endonuclease. After open complex formation, the first stage of the NER reaction is completed by dual incision of the damaged strand on both sides of the lesion. Whether TFIIH has a direct effect on the endonucleolytic activities of XPG and ERCC1-XPF was investigated using model substrates that mimic NER intermediates. These substrates consist of oligonucleotides containing partially self-complementary regions resulting in DNA duplexes of 22 bp and either a 5′- or 3′-end protruding single-stranded arm of 28 thymidine residues that will minimize the formation of secondary structures. These oligonucleotides have previously been shown to represent ideal substrates for analysis of the incision activities of both NER endonucleases (*14*, *28*) and allow to study in detail the effect of a single NER factor on XPG and ERCC1-XPF activity (*14*).

When XPG was incubated with a DNA duplex containing a 5′-end protruding single-stranded arm, a specific incision was observed in the duplex region in the strand that continues as the 5′-end protruding arm (Figure 2A, compare lanes 1 and 2). Addition of ATP had no or little effect on the reaction efficiency, while TFIIH alone did not contain any detectable nuclease activity (Figure 2A, lanes 3 and 4, respectively). Unexpectedly, when TFIIH was added to the XPG reaction

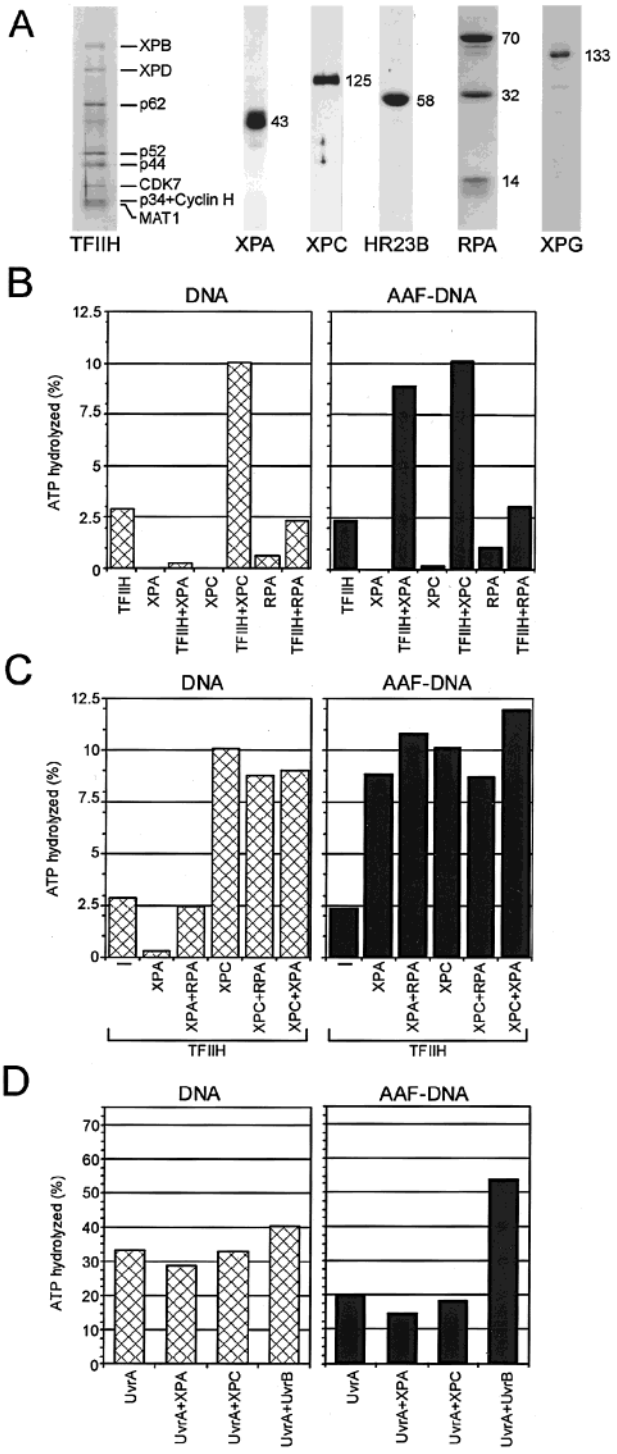

FIGURE 1: (A) Purified protein factors used in this study. SDS−polyacrylamide gels containing TFIIH stained with silver nitrate; XPA, XPC, and HR23B used to reconstitute recombinant XPC-HR23B complex as described in Materials and Methods; RPA and XPG stained with Coomassie Blue. The individual subunits of the multisubunit factors TFIIH and RPA are indicated. (B and C) Stimulation of the DNA-dependent TFIIH-associated ATPase activity by XPA and XPC-hHR23B. All values were derived from a single representative experiment to allow direct comparison. The inhibition of the ATPase activity of TFIIH by XPA in the presence of undamaged DNA (B, left panel) was not repeatedly observed in multiple experiments. (D) XPA and XPC-HR23B do not stimulate the ATPase activity of the prokaryotic UvrA protein. Reactions were carried out in the absence or presence of (*N*-acetoxy-AAF-damaged) DNA as indicated above the graphs. Hatched and filled bars represent the presence of plasmid DNA and AAF-containing plasmid DNA, respectively.

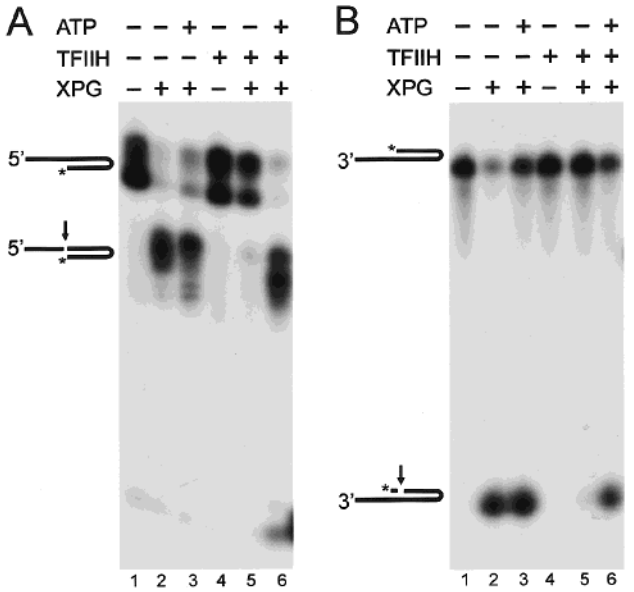

FIGURE 2: Autoradiogram of nuclease assay indicating ATP-reversible inhibition of XPG endonuclease activity by TFIIH. (A) Reactions containing 5′-end protruding-d(T)$_{28}$ substrate and factors as indicated above the lanes. The DNA substrate used displayed a heterogeneous migration pattern (lane 1), presumably due to melting and reannealing of the duplex part of the oligonucleotide during electrophoresis. The smearing of the incision product may also be due to migration conditions and/or at least in part to a 5′ to 3′ exonuclease activity associated with XPG (*35*). (B) Reactions containing 3′-end protruding-d(T)$_{28}$ substrate and factors as indicated above the lanes.

mixture in the absence of ATP, a complete inhibition of XPG nuclease activity was observed (Figure 2A, lane 5). Surprisingly, addition of ATP to the reaction containing TFIIH and XPG fully reversed the inhibitory effect of TFIIH (Figure 2A, lane 6). In these reaction mixtures, the DNA substrate was present in substoichiometric amounts as compared to both XPG and TFIIH. However, because XPG was present in a large molar excess over TFIIH (at least 5-fold), the reaction conditions strongly suggests that inhibition in the absence of ATP is mediated by binding of TFIIH to DNA, thereby blocking XPG nuclease activity.

The inhibition by TFIIH of the endonucleolytic activity of XPG did not depend on the polarity of the DNA duplex/single-stranded junction. Using a substrate containing an identical duplex region but with a 3′-end d(T)$_{28}$-protruding arm, similar results were obtained (Figure 2B). XPG efficiently incised the duplex region in the strand opposite of the 3′-end protruding arm (Figure 2B, lane 2). TFIIH completely inhibited cleavage by XPG of this substrate (Figure 2B, lane 5), which was reversed by the addition of ATP to the reaction mixture (Figure 2B, lane 6). Addition of ATPγS containing a non-hydrolyzable $\beta-\gamma$ bond could not substitute for ATP in these reactions (unpublished observations), indicating that ATP hydrolysis is required to reverse the TFIIH-mediated inhibition of XPG.

In addition to these small hairpin substrates, the inhibition of XPG endonuclease activity was also observed with other double-stranded DNA substrates containing small single-stranded loops of varying lengths (data not shown), demonstrating the specificity of the observed effects.

Effect of TFIIH and ATP on ERCC1-XPF Endonuclease Activity. The observed ATP-reversible inhibition of XPG

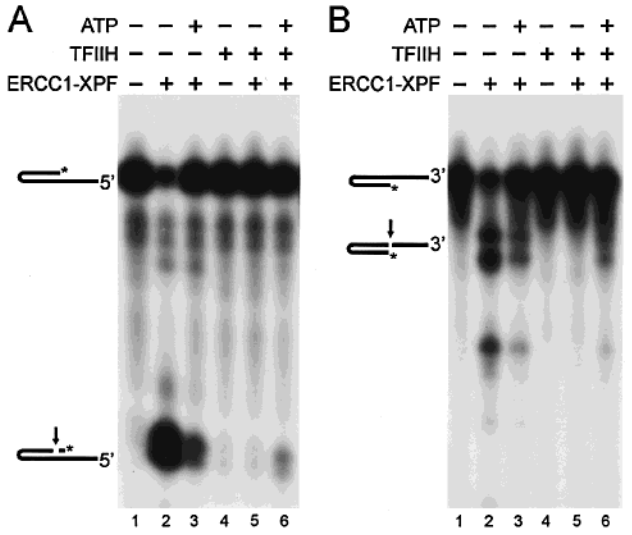

FIGURE 3: Autoradiogram of nuclease assay showing ATP-reversible inhibition of ERCC1-XPF endonuclease activity by TFIIH. (A) Reactions containing 5′-end protruding-d$(T)_{28}$ substrate and factors as indicated above the lanes. (B) Reactions containing 3′-end protruding-d$(T)_{28}$ substrate and factors as indicated above the lanes. Note that addition of ATP only (A, B; lanes 3) inhibited the nuclease reaction considerably.

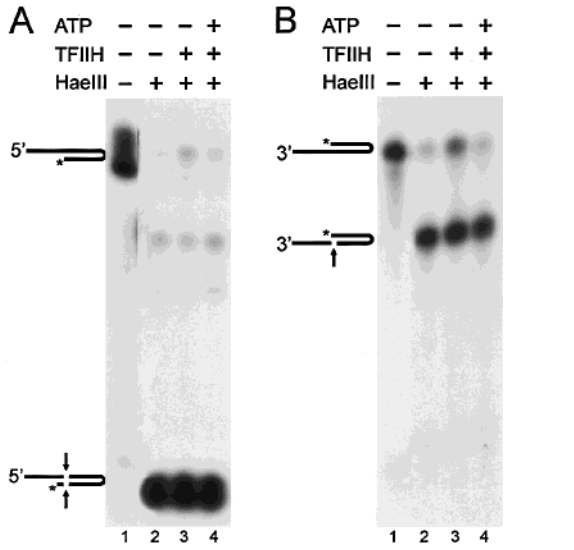

FIGURE 4: Specificity of endonuclease inhibition by TFIIH. (A) TFIIH and ATP do not affect *Hae*III endonuclease activity on the 5′-end protruding DNA substrate. (B) No effect of TFIIH and ATP on *Hae*III activity using a 3′-end protruding substrate. Note that, independent of the presence of TFIIH and/or ATP, *Hae*III was hardly able to digest the nonprotruding strand of the 3′-end protruding substrate as observed before (*14*).

activity by TFIIH prompted us to investigate whether this effect was specific for XPG or general for both NER endonucleases. Therefore, partially purified recombinant ERCC1-XPF was incubated with a 5′-end protruding-d$(T)_{28}$ DNA substrate (Figure 3A, lane 2) or with the 3′-end protruding-d$(T)_{28}$ substrate (Figure 3B, lane 2). As indicated, ERCC1-XPF was able to cut these substrates at specific sites and with opposite polarities as compared to the XPG endonuclease (compare Figures 2A and 3A and Figures 2B and 3B, lanes 2; see also ref *14*). Addition of ATP to the reaction mixtures containing ERCC1-XPF inhibited the reactions considerably (Figure 3A,B, lanes 3). This effect might—among other reasons—be due to sensitivity of ERCC1-XPF for ATP or to the increased ionic strength of the added ATP solution. Importantly, however, TFIIH was able to completely inhibit ERCC1-XPF activity in the absence of ATP (Figure 3A,B, lanes 5), while, as expected, TFIIH alone did not have any effect on the DNA substrates (Figure 3A,B, lanes 4). Interestingly, despite its inhibitory effect and the presence of TFIIH, addition of ATP to reaction mixtures containing TFIIH and ERCC1-XPF induced a partial but significant restoration of the nuclease activity as compared to the level of the control reaction containing ATP and ERCC1-XPF only (Figure 3A,B compare lanes 6 and 3). Addition of ATPγS did not reverse the inhibition by TFIIH of ERCC1-XPF (unpublished observations), indicating that hydrolysis of the $\beta-\gamma$ bond of ATP is also important for the relief of inhibition by TFIIH of ERCC1-XPF.

These findings indicate that ATP-reversible inhibition by TFIIH is a general mechanism for both NER endonucleases. To exclude the possibility that the inhibition of the endonuclease activities by TFIIH was due to aspecific blocking of the DNA duplex/single-stranded junction, the availability of a restriction enzyme site (GGCC; *Hae*III) at the last four base pairs of the duplex region was determined. When *Hae*III was added to the reaction mixture containing the 5′-end protruding-d$(T)_{28}$ DNA substrate and TFIIH either in the presence or in the absence of ATP restriction enzyme, digestion of the DNA was unaffected (Figure 4A). Similar results were obtained in the presence of the 3′-end protruding-d$(T)_{28}$ DNA substrate (Figure 4B).

Because the incision sites of XPG and ERCC1-XPF and the availability of the *Hae*III restriction enzyme site appeared unchanged in the presence of both TFIIH and ATP, stable unwinding of the DNA duplex region does not occur to a significant extent under the conditions used. Thus, the ATP-dependent reversal of inhibition is caused by a novel role of TFIIH and ATP beyond DNA unwinding.

DISCUSSION

Besides an essential cellular transcription factor, TFIIH is a core constituent of NER involved in helix unwinding. Many protein interactions described between NER factors appear not stable and may represent transient interactions (*25*, *29*). In this paper, the functional effect of repair factors on TFIIH activity and vice versa was analyzed revealing stimulatory roles for XPA and XPC-HR23B on the DNA-dependent ATPase activity of TFIIH and a dependence of the dual incision by the XPG and ERCC1-XPF complex on ATP hydrolysis by TFIIH.

Both the XPC-HR23B complex and XPA have affinity for TFIIH and are able to recruit TFIIH preferentially to damaged DNA (*30*−*33*). The significance of these observations is extended by the findings presented in this paper, which show that XPC-HR23B and XPA can strongly stimulate the TFIIH-associated ATPase. XPC-HR23B binds with high specificity to DNA damage (*7*), but nonetheless, the complex does not confer specificity to the TFIIH-associated ATPase for damaged DNA, which may be due to the strong binding capacity of the XPC complex to undamaged double-stranded DNA as well (*30*, *34*). In contrast, the fact that XPA only stimulates the ATPase activity of TFIIH in the presence of damaged DNA may

suggest that the interaction of XPA and nondamaged DNA is simply not stable enough or is not able to induce the proper protein−DNA conformation to further activate the TFIIH-associated ATPase. The stimulation of the ATPase activity of TFIIH by XPC-HR23B and XPA is in agreement with the notion that both proteins are actually involved in the unwinding step either sequentially or simultaneously. A possible mechanism by which XPC-HR23B and XPA stimulate the ATPase activity is by tethering the DNA-dependent ATPase subunits XPB and XPD to DNA. Alternatively, or in addition, XPC-HR23B and XPA may alter the conformation of the TFIIH complex into an activated form.

A second pair of interactions involving TFIIH surprisingly reveals that this complex inhibits the endonuclease activities of both XPG and ERCC1-XPF. Physical interactions between TFIIH and XPG have been reported using both human and yeast proteins (*36*, *37*) but not between TFIIH and ERCC1-XPF. On the basis of the reaction stoichiometry, the specific inhibition is likely mediated via ternary complex formation on DNA. Addition of ATP resulted in relief of repression of nuclease activity via a mechanism distinct from TFIIH-mediated DNA unwinding, because the incision sites of the model substrates were not clearly altered, and the availability of a restriction enzyme site was not affected by the presence of TFIIH and ATP. It is unlikely that phosphorylation is involved in the reversal of repression of XPG and ERCC1-XPF activity. Although TFIIH can readily phosphorylate XPG, ERCC1-XPF is not recognized as a substrate by the TFIIH kinase in vitro (G.S.W. and J.H.J.H., unpublished observations). Furthermore, the kinase catalytic subunit CDK7 is dispensable for dual incision in defined reactions (*5*, *6*).

Using model substrates that mimic NER intermediates, we show that, in contrast to RPA (*14*), TFIIH influences XPG and ERCC1-XPF activity independent of the polarity of the DNA duplex/single-stranded junction of the substrate. This suggests that TFIIH binds to both the damaged and the undamaged strand around the DNA damage or to a double-stranded DNA region outside the unwound area. A possible mechanism to explain the observed effect is that ATP induces a conformational change in the nuclease-TFIIH-DNA ternary complex, involving displacement of TFIIH from the single-strand/double-strand junctions thereby relieving inhibition of nuclease activity. The inhibitory effect of TFIIH on incisions by XPG and ERCC1-XPF may prevent inadvertent incisions of these structure-specific endonucleases prior to full opening of the DNA helix.

A novel role for TFIIH and ATP beyond DNA unwinding provides a rationale for at least three previously published unexplained observations. (i) In XP-B extracts derived from XP11BE cells containing a mutation that affects the C-terminal 40 amino acids of the XPB subunit, full opening around a DNA lesion is observed, but the formation of the 5′ incision is defective (*9*). Together with the findings presented in this paper, these data suggest that the carboxy terminus of XPB is important for the ATP-dependent relief of ERCC1-XPF inhibition by TFIIH. (ii) NER substrates with a DNA lesion located in a premelted region still require TFIIH for dual incision (*10*). (iii) The formation of the 3′ and 5′ incision made by XPG and ERCC1-XPF, respectively, are closely coupled in time, and kinetic experiments indicate that the 3′ incision is made before the 5′ incision (*6*, *9*). These observations are extended by the data presented in this paper, which suggest that the formation of incisions by XPG and ERCC1-XPF are both regulated by a mechanism distinct from DNA unwinding involving TFIIH and ATP.

Taken together, the findings presented in this paper reveal additional roles for TFIIH and ATP, which have important implications for the mechanism of dual incision and may involve conformational changes mediated by the TFIIH-associated ATPase activity distinct from DNA unwinding.

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