

DNA Damage Recognition by XPA Protein Promotes Efficient Recruitment of Transcription Factor II H*

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The human basal transcription factor IIIH (TFIIH) is an essential component of the nucleotide excision repair machinery. TFIIH is required for reaction steps concomitant with or prior to the formation of dual incisions in the damaged DNA strand. To understand the mechanism underlying the recruitment of TFIIH to DNA damage sites we have analyzed i) the direct affinity of TFIIH for damaged or undamaged DNA and ii) the interaction of TFIIH with XPA-DNA complexes, formed using unirradiated or UV-irradiated DNA.

Filter binding assays showed that TFIIH has some affinity for the DNA, but in contrast to XPA, does not show any preference for UV-irradiated DNA. Pull-down experiments demonstrated that TFIIH binds to XPA-DNA complexes in an UV damage-dependent manner by a direct protein-protein interaction with XPA. We propose that an enhancement of the affinity of XPA protein for TFIIH could arise from conformational changes of XPA when it binds to UV lesions on the DNA.

Transcription initiation of protein coding genes and nucleotide excision repair (NER)¹ of damaged DNA were shown to be connected through the dual action of TFIIH, a multisubunit protein complex that contains several enzymatic activities (reviewed in Refs. 1 and 2). Although the protein composition and the nature of the different subunits are almost completely determined (Ref. 3 and references therein), relatively little is

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¹ The abbreviations used are: NER, nucleotide excision repair; TFIIH, transcription factor IIIH; XP, xeroderma pigmentosum; RPA, replication protein A; ERCC, excision repair cross-complementing; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; XPA, XPB, and XPD define the products of xeroderma pigmentosum complementation group A, B, and D genes, respectively.

known concerning the precise functions of TFIIH in both mechanisms. However, genetic studies in yeast (4, 5) and Chinese hamster ovary cells (6), as well as the association of mutations in XPB and XPD with the human disorders xeroderma pigmentosum (XP), Cockayne syndrome, and trichothiodystrophy (7, 1), demonstrate the crucial importance of TFIIH in cellular DNA metabolism.

During transcription initiation, TFIIH is thought to be recruited to a promoter, after the formation of the preinitiation complex containing TFIID, TFIIB, TFIIF, and RNA polymerase II on the TATA box consensus sequence (8). Once associated with this complex, TFIIH is likely to function through its ATP-dependent helicase subunits, which may facilitate the opening of the promoter region (9) to allow the reading of the DNA. The cyclin dependent-kinase activity of TFIIH phosphorylates the carboxyl-terminal domain of RNA polymerase II in a reaction that has been proposed to activate the elongation process (10, 11). During NER, TFIIH is thought to be recruited to the damage site at an early step of the repair process (12–14). The initial recognition of DNA lesions is likely to involve a nucleoprotein-DNA complex consisting of XPA, a zinc finger protein with some specificity for UV- or chemical carcinogen-damaged DNA (15–19), RPA (20–24), and probably other factors. TFIIH may then be involved in the formation of a preincision complex through an interaction with the COOH terminus of XPA (25). The precise role played by TFIIH during the early reaction steps of NER is not yet certain, but the ATP-dependent helicase activities of the XPB and XPD subunits (26–29, 6) may facilitate the formation of an open DNA complex, which precedes the formation of dual incisions in the damaged DNA strand (30).

In the present study, we have analyzed the mechanism of TFIIH recruitment to damaged DNA. We found that the binding of XPA to damaged DNA is a prerequisite for the efficient recruitment of the TFIIH complex.

MATERIALS AND METHODS

Plasmid Constructs—Construction of the expression vector pGEX-XPA has been described elsewhere (24). Construction of pGEX-XPA.CΔ46 was performed by integrating in pGEX-2TK an *EcoRI* fragment containing the complete cDNA sequence of the mutated XPA protein from the XP390S patient.² The mutation consists in a C to T transition at nucleotide 682 altering the Arg²²⁸ codon (CGA) to a nonsense codon (TGA) (31) and resulting in a truncated XPA protein (32).

Proteins and Antibodies—GST, GST-XPA, and GST-XPA.CΔ46 were prepared as already described (24). Preparation of TFIIH and of antibodies against the various TFIIH subunits have been described elsewhere (3). Whole cell-free extracts were prepared from HeLa cells according to Manley *et al.* (33), with minor modifications.

DNA Preparations—pUC19 DNA was linearized by restriction with *EcoRI*. Partial filling of recessed 3'-ends was carried out using [α -³²P]dATP and the Klenow fragment of *Escherichia coli* DNA polymerase I. Aliquots were irradiated on ice with a germicidal lamp with peak output at 254 nm at doses up to 20 KJ/m². A 48-mer double strand DNA containing an internal stretch of 8 thymines was synthesized by polymerase chain reaction and irradiated as described for pUC19 DNA. A 879-base pair DNA fragment was obtained by *Bam*HI/*Ssp*I restriction of pUC309 (derived from pUC19) plasmid DNA. Aliquots were irradiated at 1 KJ/m². Damaged and undamaged DNA fragments were ³²P-labeled by extension with the Klenow fragment of DNA polymerase I.

Filter Binding Assay—1 ng of the ³²P-labeled 879-base pair DNA

² T. Kobayashi, S. Nocentini, Y. Nakatsu, and K. Tanaka, Manuscript in preparation.

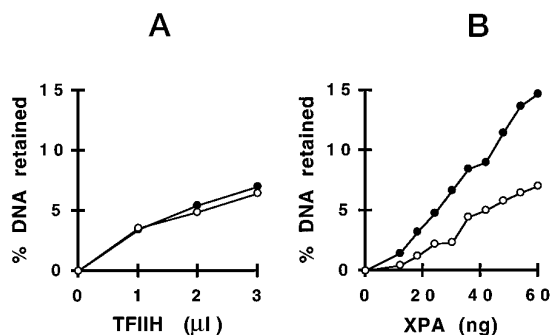


FIG. 1. XPA and TFIIH binding to undamaged and UV-damaged DNA. DNA binding activities of TFIIH (A) and of GST-XPA protein (B) were examined by a filter binding assay using unirradiated DNA (○) or DNA irradiated with 1 KJ/m² (●). The GST tag does not interact with DNA (data not shown).

probe (approximately 5,000 cpm) was incubated with various quantities of GST-XPA recombinant protein or of highly purified TFIIH (the hydroxyapatite-eluted fraction, which is the last step of our purification procedure (see Ref. 3 and Fig. 3C)), in 20 μ l of a buffer containing 50 mM Tris-HCl, pH 7.9, 10% glycerol, 0.1 mM EDTA, 0.5 mM dithiothreitol, 50 mM KCl, 5 mM MgCl₂, 60 μ g/ml bovine serum albumin, and 0.5 μ g poly(dG-dC), for 30 min at 30 °C. The reaction mixtures were then loaded on a nitrocellulose membrane by using a hybridot device. After washing with a buffer containing 50 mM Tris-HCl, pH 7.9, 10% glycerol, 0.1 mM EDTA, 50 mM KCl, the labeled probe retained on the membrane was quantified with a PhosphorImager (Molecular Dynamics).

Protein-Protein Interaction Assay—The interaction of TFIIH with wild type or mutant GST-XPA fusion proteins was analyzed by pull-down experiments. GST-XPA fusion proteins (500 ng) were adsorbed to glutathione-Sepharose beads (10 μ l) directly or after incubation with untreated or UV-irradiated linearized pUC19 DNA for 1 h at 4 °C in a buffer containing 40 mM Hepes-NaOH, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol. After washing, the beads were incubated with aliquots of HeLa whole cell extract (equivalent to 5 \times 10⁵ cells/assay) or with purified preparations of TFIIH for at least 2 h at 4 °C with mild agitation in a buffer containing 40 mM Hepes-NaOH, pH 7.5, 100 mM KCl, 0.2 mM EDTA, 0.4% Nonidet P-40, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 0.5 μ g/ml pepstatin, 0.5 μ g/ml leupeptin. The beads were then extensively washed with the same buffer. The bound proteins were extracted by boiling in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.004% bromophenol blue, 0.1 M dithiothreitol), separated by SDS-PAGE, and analyzed by immunoblotting using the Aurora™ Western blot chemiluminescent detection system (ICN).

RESULTS

TFIIH Does Not Bind Preferentially to Damaged DNA—The presence of some zinc finger motifs, including a TFIIA-like motif, as well as some CX2C cystein-rich motifs in p44, one of the subunits which forms the core of TFIIH (34), strongly suggested that TFIIH directly binds DNA. We tested this possibility using a nitrocellulose filter binding assay, supposing that TFIIH, because of its involvement in both transcription and DNA repair, might interact with undamaged and damaged DNA, respectively. Highly purified TFIIH (the hydroxyapatite-eluted fraction from our purification procedure) was thus incubated with an UV-irradiated or untreated ³²P-labeled pUC309 DNA fragment as described under “Materials and Methods,” and the interaction was estimated by the ability of TFIIH to retain labeled DNA on a nitrocellulose filter. As shown on Fig. 1A, TFIIH interacted equally with damaged and undamaged DNA, which demonstrates that TFIIH *per se* has no specificity for damaged DNA. This binding is likely to occur through the zinc finger motif, since mutations in this sequence completely abolish the binding of the recombinant p44 subunit on any DNA.³ On the contrary, when the same experiment was performed with the XPA recombinant protein, we observed that

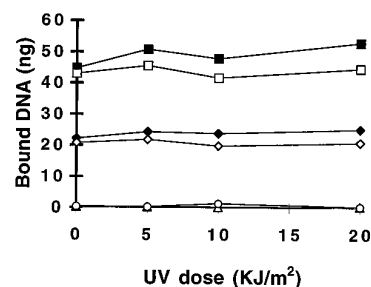


FIG. 2. Measurement of untreated or UV-irradiated DNA complexed with XPA in pull-down experiments. 25 (◆, ◊, △) or 50 ng (■, □, ○) of end-labeled pUC19 DNA were incubated for 1 h at 4 °C with 500 ng of GST-XPA or GST-XPA.CΔ46 proteins before adding glutathione-Sepharose beads and continuing the incubation for 1 more h, according to our standard procedure. After extensive washing, the amount of DNA retained by the proteins bound to the affinity support was determined by counting the radioactivity precipitated relatively to that employed. ◆, ■, GST-XPA; ◊, □, GST-XPA. CΔ46; △, ○, GST tag.

the retention of UV-damaged DNA was higher than the retention of undamaged DNA (Fig. 1B), a point that was previously established using gel shift retention and filter binding assays (16, 18, 19). At the present stage of our investigations, it is difficult to compare the affinity constants of both factors toward any kind of DNA, since the exact stoichiometry of TFIIH has not yet been established.

TFIIH Binds to XPA Complexed with DNA in an UV Damage-dependent Manner—Since TFIIH was demonstrated to participate in the early steps of NER, as do several damage recognition factors, including the XPA protein (12–14), it was of interest to test how its recruitment to sites of lesions could be selectively promoted. Therefore, we designed experiments in which a fixed amount of GST-tagged XPA was preincubated in the presence or absence of increasing amounts of *Eco*RI-linearized pUC19 DNA, either untreated or previously UV-irradiated at different doses (5, 10, and 20 KJ/m²), before being adsorbed onto affinity glutathione-Sepharose beads. DNA concentrations were chosen so that UV-irradiated DNA, as well as undamaged DNA, were in limited amounts with regard to the binding capacities of the XPA protein in the assay. Consequently, and as verified using end-labeled pUC19 DNA, almost all of a given quantity of unirradiated or UV-irradiated DNA was found complexed to the GST-XPA protein immobilized on the beads (Fig. 2). We also checked that the amount of GST-XPA protein bound to the glutathione-Sepharose beads was the same irrespective of the preincubation with either UV-irradiated or unirradiated DNA (data not shown). This implies that the only variable between the different XPA-DNA complexes (formed with 25 ng of DNA, for example) was the number of UV lesions on the DNA. The various samples were subsequently incubated with aliquots of HeLa whole cell extract. After extensive washing with the same buffer containing 100 mM KCl and 0.4% Nonidet P-40, the polypeptides retained on the affinity support were resolved by SDS-PAGE followed by immunoblotting using antibodies directed toward the p62 and cyclin H subunits of TFIIH (Fig. 3A). Several observations can be drawn from these experiments. First, TFIIH present in HeLa whole cell extracts is significantly retained on the affinity support only when GST-XPA has bound pUC19 DNA; in fact, GST-XPA alone is only able to retain a very low amount of TFIIH (Fig. 3A, compare lane 2 and lanes 3–5). Second, the amount of TFIIH retained depends upon the presence of DNA and the number of lesions induced by UV irradiation. Indeed, increasing concentrations of damaged DNA, as well as increasing UV doses to DNA, resulted in a corresponding increase in the capacity of preformed GST-XPA-DNA complexes to retain TFIIH (Fig. 3A, compare lanes 3–5 and 6–14). Assayed as a control, TFIIIF α , a

³ V. Moncollin, S. Humbert, and J.-M. Egly, unpublished results.

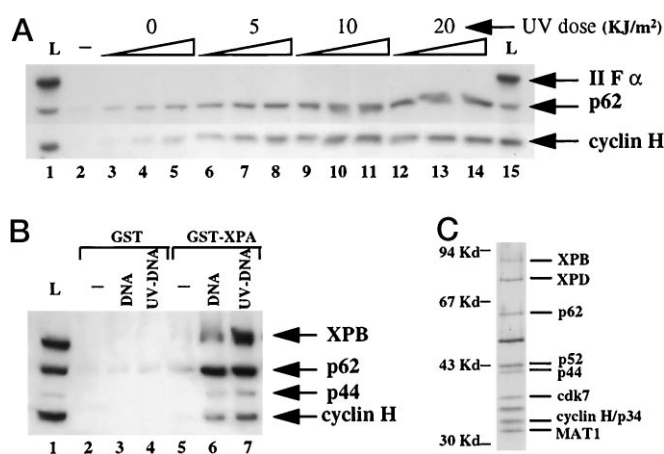


FIG. 3. TFIIF binding to XPA and to XPA complexed to untreated or UV-irradiated DNA. *A*, GST-XPA protein was incubated with various quantities of unirradiated or UV-irradiated linearized pUC19 DNA (0, 25, 50, or 100 ng) and then adsorbed to glutathione-Sepharose beads. After washing, the beads were incubated with aliquots of HeLa whole cell extract. The beads were then extensively washed and bound proteins extracted by boiling in SDS sample buffer, separated by SDS-PAGE, and analyzed by immunoblotting with anti-p62, anti-cyclin H, and anti-TFIIF α antibodies. *B*, pull-down experiments were performed using GST-XPA bound or not to a 48-mer DNA unirradiated or UV-irradiated at 20 KJ/m² and highly purified TFIIF (Fig. 3C). GST was employed as control. The interaction was revealed by immunoblotting the retained TFIIF with different antibodies against several TFIIF subunits. *C*, analysis of the hydroxyapatite-eluted TFIIF fraction by SDS-PAGE and silver staining of the gel. The molecular mass size markers are indicated on the left, and the TFIIF subunits are indicated on the right. Two bands are polypeptides that comigrate with the 9 TFIIF subunits presently identified through the seven steps of our purification procedure (3).

transcription factor that is known to associate with RNA polymerase II for transcription initiation, as well as for elongation, was observed not to bind with GST-XPA whether or not the latter was associated with DNA (Fig. 3A). Taken together, these data indicate that TFIIF present in HeLa whole cell extracts interacts with XPA-DNA complexes as a function of the number of lesions on the DNA.

To establish whether the binding of TFIIF on GST-XPA·(UV-damaged) DNA complexes is direct or mediated by other polypeptides present in HeLa cell crude extracts, we incubated highly purified TFIIF (Fig. 3C) with GST-XPA, which had previously been incubated with either UV-irradiated (at a dose of 20 KJ/m²) or nonirradiated 48-mer DNA fragments (containing a stretch of 8 thymines). The detection of increased amounts of the XPB, p62, p44, and cyclin H subunits of TFIIF associated with complexes of GST-XPA and UV-irradiated DNA (Fig. 2B, compare lanes 6 and 7) suggests that purified TFIIF preferentially binds to these complexes. This association is specific, since TFIIF was not retained by the GST tag (lanes 2–4). Also, we can observe again that TFIIF binds weakly to GST-XPA in the absence of DNA (lane 5). This experiment thus indicates that the interaction of TFIIF with XPA-DNA complexes is direct and not mediated by other factors.

Since TFIIF has some affinity for DNA (Fig. 1A), the question may be raised whether the binding of TFIIF to XPA-DNA complexes involves a direct protein-protein interaction with XPA or results from TFIIF binding to the DNA. To answer this issue, we have compared the binding of TFIIF to protein-DNA complexes formed using the wild type GST-XPA protein or a mutant GST-XPA.C Δ 46 protein. The C Δ 46 deletion (32), which results from the mutation altering the Arg²²⁸ codon (CGA) to a non-sense codon (TGA) (31), does not affect the DNA binding domain of the protein (35), but is crucial for NER, because the patient homozygous for this mutation showed an XP phenotype

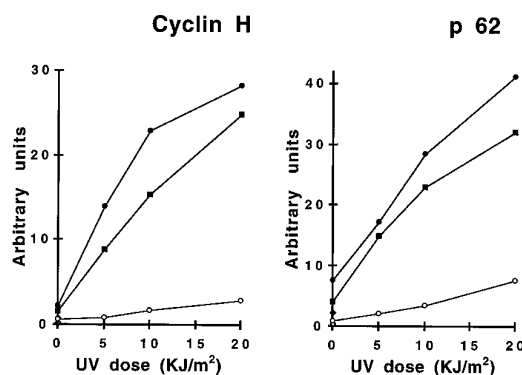


FIG. 4. UV damage dependence of TFIIF binding to XPA or XPA.C Δ 46-DNA complexes. Experiments were carried out as described in the legend to Fig. 3, and results are expressed as arbitrary units as a function of the UV irradiation given to linearized pUC19 DNA employed at 25 or 50 ng/assay. Symbols for both panels: \blacklozenge , GST-XPA alone; \blacksquare , GST-XPA-25 ng of DNA; \bullet , GST-XPA-50 ng of DNA; \diamond , GST-XPA.C Δ 46 alone; \circ , GST-XPA.C Δ 46-50 ng of DNA.

(31, 32). Either GST-XPA or GST-XPA.C Δ 46 fused proteins were incubated in two steps as described above: first with damaged or undamaged DNA and second with HeLa whole cell extract, and the presence of TFIIF on the affinity support was analyzed by SDS-PAGE followed by immunoblotting. In these experimental conditions and using end-labeled pUC19 DNA, we first observed that the mutant GST-XPA.C Δ 46 protein still possesses the capacity to bind DNA, with an efficiency close to that of wild type GST-XPA protein (Fig. 2). We also verified that the binding of GST-XPA.C Δ 46 alone or of GST-XPA.C Δ 46 complexed with damaged or with undamaged DNA to glutathione-Sepharose beads was the same. However, as observed on the immunoblots using anti-p62 and anti-cyclin H antibodies (Fig. 4, compare GST-XPA.C Δ 46 curve to GST-XPA curves), TFIIF was retained to a much lower extent on GST-XPA.C Δ 46-DNA complexes than on GST-XPA-DNA complexes, independent of the level of DNA damage. Furthermore, another truncated XPA protein MF122, composed of 122 amino acid residues spanning from position 98 to 219 and which still possesses DNA binding activity (35), did not significantly interact with TFIIF in the presence of undamaged or damaged DNA (data not shown). These results indicate that TFIIF binds directly to XPA engaged in a protein-DNA complex and that the carboxyl-terminal domain of XPA is important for this interaction. The latter finding is consistent with a previous report (25).

DISCUSSION

Active processing of DNA damage by NER is dependent on the ability to recognize DNA modifications in the genome and on the efficiency with which repair factors are recruited to the site of DNA damage. Evidence is accumulating that selectivity in DNA damage recognition is achieved through the assembly of nucleoprotein complexes at the sites of lesions. A first level of specificity probably arises through the DNA damage binding properties of XPA in tight association with RPA. *In vitro* experiments have shown that XPA (Refs. 16, 18, and 19 and our present result) and RPA (23) have a moderately higher affinity for damaged DNA over undamaged DNA in isolation and a considerably enhanced preferential binding of both proteins when the two proteins are present (22, 23). More specificity in damage recognition may be gained by interaction of XPA with ERCCI (36–38), which was also shown to increase the affinity of XPA for damaged DNA (39). These cooperative protein-protein-DNA interactions, possibly stabilized by other factors, are likely to result in the accumulation of XPA in “closed” preincision complexes at sites of lesions. TFIIF is thought to

enter the assembly at this point of the repair process.

The experiments presented here provide insight into the mechanism by which TFIID is recruited to DNA damage. We have observed that TFIID binds much more efficiently to GST-XPA-UV-damaged DNA complexes than to GST-XPA-undamaged DNA or GST-XPA.C446-UV-damaged DNA complexes. Thus, taken together, these data indicate that TFIID has no marked preference for binding to damaged DNA by itself and is instead recruited to XPA-DNA complexes mainly via an interaction with the COOH terminus of XPA whose affinity for TFIID appears to be damage recognition-dependent. This finding that UV damage to DNA potentiates the affinity of XPA for TFIID has interesting implications for the functions of TFIID *in vivo*. The discovery that some components of the NER system participate in other important metabolic processes, *i.e.* transcription for TFIID and replication for RPA, suggests that there might be mechanisms for shifting from one pathway to another. A possible molecular strategy could be based on changes of affinity between the different interacting proteins. Our results suggest that XPA in binding to UV-damaged DNA undergoes conformational changes enabling a much more efficient interaction with TFIID. The XPA-RPA and, possibly, XPA-ERCC1 cooperativities in the recognition of damaged DNA, together with the resulting increase of XPA affinity for TFIID, may confer the specificity and the efficiency necessary to sequester TFIID away from actively transcribed promoters to NER preincision complexes and, in particular, may direct TFIID to damage sites in nontranscribed regions of the genome. Once recruited to the lesion by XPA, TFIID may also bind to the DNA by its p44 or p34 subunits (34) and lead to open complex formation via its ATP-dependent helicase activities (30) prior to endonucleolytic cleavage of the damaged DNA strand by structure-specific nucleases XPG (40) and XPF-ERCC1 (41).

Although many studies have demonstrated that the NER system can recognize and repair a wide range of chemically and structurally distinct lesions, the efficiency of their removal varies greatly. Further development of our work will try to establish if the affinity of XPA for TFIID is influenced by different types of DNA lesions and if this may be correlated with the rates of their repair.

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