RNA Polymerase II Elongation Complexes Containing the Cockayne Syndrome Group B Protein Interact with a Molecular Complex Containing the Transcription Factor IIH Components Xeroderma Pigmentosum B and p62*

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Transcription factor IIH (TFIIH) is involved both in transcription initiation by RNA polymerase II and in nucleotide excision-repair. Nucleotide excision-repair occurs at higher rates in transcriptionally active regions of the genome. Genetic studies indicate that this transcription-coupled repair is dependent on the Cockayne syndrome group A and B proteins, as well as TFIIH subunits. Previous work indicated that Cockayne syndrome group B interacts with RNA polymerase II molecules engaged in ternary complexes containing DNA and RNA. Evidence presented here indicates that this complex can interact with a factor containing the TFIIH core subunits p62 and xeroderma pigmentosum subunit B/excision repair cross-complementing 3. The targeting of TFIIH or a TFIIH-like repair factor to transcriptionally active DNA indicates a potential mechanism for transcription-coupled repair in human cells.

TFIIH† is a complex factor capable of multiple functions (for review, see Hoeijmakers et al. (1)). It contains approximately nine subunits, although considerable compositional variability has been reported (2–5). TFIIH contains multiple enzymatic activities, including two distinct DNA helicase activities encoded by the xeroderma pigmentosum (XP) B/excision repair cross-complementing (ERCC) 3 and XPD/ERCC2 subunits (6, 7), and a kinase activity capable of phosphorylating the pol II large subunit carboxyl-terminal domain (CTD) (5, 8). In addition to XPD and XPB, several other TFIIH subunits are important NER factors (9–12).

Under most conditions, TFIIH is required for pol II transcription initiation. The need for TFIIH correlates with the well-documented pol II requirement for the energetic β-γ phosphoanhydride bond of ATP (13, 14). Negative supercoiling allows some promoters to initiate transcription without TFIIH in vitro (15), and concurrently circumvents the energetic requirement for ATP (16). Templates bearing heteroduplex start sites also eliminate the requirement both for TFIIH and for hydrolysis of the ATP β-γ phosphoanhydride bond (17–19), suggesting that during pol II initiation, TFIIH uses the energy of ATP hydrolysis to promote local DNA unwinding at the transcription initiation site. This melted DNA intermediate is termed an "open complex." An open complex is also formed during NER in a manner that is dependent on the TFIIH helicases and ATP (20, 21). The NER open complex appears to serve as a substrate for the site-specific endonucleases XPG and XPF. TFIIH is also implicated in post-initiation events, such as promoter escape and pol II elongation (22).

Recent studies have suggested that TFIIH exists in multiple forms (23, 24). In a transcriptionally active form, a set of common "core" subunits, consisting of XBP/ERCC3 (yeast SSL2), XPD/ERCC2 (RAD3), p62 (TFB1), p52 (TFB2), p44 (SSL1), and p34 (TFB4) (reviewed in Refs. 25 and 26), associate with a cyclin-Cdk complex that contains Cdk7/MO15 (yeast KIN28), cyclin H (CCL1), and MAT1 (TFB3). This kinase complex was previously equated with an activity, Cdk-activating kinase (known as CAK), that could phosphorylate and activate other Cdk molecules. Although evidence indicates that this complex may not be the physiologically relevant form of Cdk-activating kinase in yeast (27), the Cdk7 complex may nevertheless fulfill this role in metazoans (28). Another form of TFIIH is associated with other NER proteins and has been termed the "repairosome" (2, 29, 30).

A number of human disorders stem from defects in TFIIH subunits. For example, mutations in several TFIIH subunits can lead to XP, a condition typified by extreme UV sensitivity, a high incidence of cancer, and defective NER. Specific mutations in the TFIIH subunits XBP and XPD, as well as the TFIIH-associated repair factor XPG, cause XP with manifestations of Cockayne’s syndrome (CS), a complex disorder characterized by mental retardation, small stature, neurological defects, and UV sensitivity (reviewed in Lehman (31)). Cells taken from CS patients are sensitive to various DNA-damaging agents, such as UV radiation and the UV-mimetic agent N-acetoxy-2-acetylaminofluorene (32, 33), and they fail to recover the ability to synthesize RNA after exposure to UV (34, 35). CS cells are also defective in TCR, as transcriptionally active genes do not repair transcriptionally active genes at higher rates than the surrounding DNA (35–37). Interestingly, the majority of CS patients do not harbor mutations in TFIIH subunits, but rather in two genes termed CSA and CSB/ERCC6 (38, 39). Speculation has focused on CSA and CSB as eukaryotic transcription-repair coupling factors (1, 25, 40). In vitro evidence indicates that CSA and CSB interact with one another, that CSA interacts with the TFIIH p44 subunit (39), and that CSB interacts with the NER damage recognition factor XPA (41) and the TFIIH-associated factor XPG (42).

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TFIIH is itself an important repair protein, as both the yeast and mammalian factors were shown to complement NER-defective cell extracts (11, 43). The yeast TFIIH was shown to complement a rad3 extract, whereas the isolated Rad3 protein could not (43), indicating that Rad3 operated only in the context of the intact complex. These facts indicate that recruitment of TFIIH to sites of DNA damage may be an important step in the repair process. This supposition is substantiated by the finding that TFIIH can be recruited to DNA lesions by the XPA damage recognition protein in the absence of TFIIIE, a factor that is important for recruiting TFIIH during formation of a transcription initiation complex (44, 45). Although TFIIH plays a key role in NER, experiments suggest that TFIIH is lost from the pol II complex early in the transcription process and does not associate with the elongating polymerase (46). No evidence for re-recruitment of TFIIH to elongating pol II complexes has thus far been presented, and the potential for re-recruitment of TFIIH in TCR remains unexplored. More specifically, it is not known whether TFIIH recruitment to TCR follows a transcription-like pathway, in which TFIIH would play a role, a repair-like pathway, in which TFIIH is likely recruited by XPA, a combination of both pathways, or a mechanism in which TFIIH is recruited by other proteins.

Previous work described an interaction between transcription complexes containing pol II, DNA, RNA, and the purified CSB/ERCC6 protein (47). The complexes that interact with CSB were shown to be engaged in productive transcription. This interaction requires hydrolysis of the ATP β-γ phosphohydryde bond. In this study, purified recombinant human CSB, an oligo(dC)-tailed template, immunopurified pol II, and partially purified TFIIH are used to explore the potential of the CSB protein to interact simultaneously with transcribing pol II molecules and with TFIIH. The results indicate that a pol II-CSB-DNA-RNA quaternary complex has the ability to recruit TFIIH or a related factor, providing a plausible mechanism for TCR in mammalian cells.

MATERIALS AND METHODS

Expression of CSA, CSB, and RNA Polymerase II—CSA and CSB were expressed and purified as described previously (47). Pol II was immunopurified from HeLa nuclear pellets using a combination of two protocols (48, 49).

Purification of TFIIH—TFIIH was purified from HeLa nuclear extracts as follows. 40 ml of extract were applied to a 35-ml phosphocellulose column (Amersham Pharmacia Biotech) equilibrated in buffer D (20 mM HEPES, pH 7.9, 1.0 mM EDTA, 20% glycerol, 1.0 mM dithiothreitol, 0.5 mM phenylmethylsulfonil fluoride) containing 0.1 M KCl. The column was washed with buffer containing 0.3 M KCl and eluted in a gradient of buffer D containing 0.3 to 1.0 M KCl. Eluted TFIIH was monitored by immunoblotting of fractions using monoclonal antibodies against the XBP, XPD, and p62 subunits (gifts of D. Reinberg and J.-M. Egly). TFIIH-containing fractions were pooled, avoiding those that contained high amounts of the p56 subunit of TFIIE as judged by immunoblotting of fractions using monoclonal antibodies against the XPB, XPD, and p62 subunits of TFIIH, and was judged free of TATA-binding factor that is important for recruiting TFIIH during formation of a transcription initiation complex (44, 45). Although TFIIH plays a key role in NER, experiments suggest that TFIIH is lost from the pol II complex early in the transcription process and does not associate with the elongating polymerase (46). No evidence for re-recruitment of TFIIH to elongating pol II complexes has thus far been presented, and the potential for re-recruitment of TFIIH in TCR remains unexplored. More specifically, it is not known whether TFIIH recruitment to TCR follows a transcription-like pathway, in which TFIIH would play a role, a repair-like pathway, in which TFIIH is likely recruited by XPA, a combination of both pathways, or a mechanism in which TFIIH is recruited by other proteins.

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Preparation of C-tailed Template/Probes—Use of these templates was exactly as described previously (47).

RESULTS

TFIIH Does Not Interact with pol II Ternary Complexes—A previous study used a double-stranded DNA template bearing an oligo(dC) tail and highly purified preparations of pol II to analyze the biochemistry of the CSB protein (47). The probe can be used in both transcription and gel mobility-shift assays. Furthermore, the two assays can be combined in a labeled RNA-based gel mobility-shift assay that visualizes pol II complexes containing labeled RNA.

In Fig. 1, partially purified TFIIH was added to the end-labeled probe in reactions omitting or containing a highly purified preparation of pol II. This partially purified TFIIH preparation was purified through four chromatographic steps, active for transcription, and free of the other general transcription factors, XFG, and pol II (see “Materials and Methods”). In the absence of pol II, TFIIH did not interact significantly with the probe either in the absence (lane 2) or the presence (lane 3) of a full complement of NTPs. A preparation of mostly hypophosphorylated pol II (pol IIA) interacted with the labeled probe, producing a discreet shift (lane 4). A monoclonal antibody against the pol II CTD (SWG16) (49) supershifted the pol II complex (lane 5, brackets), indicating that the shift is due to pol II. TFIIH did not significantly interact with the bound pol II (lane 6). In the presence of pol II, TFIIH, and NTPs a new band appeared, corresponding to the hyperphosphorylated (pol IIO) form of the polymerase (lane 7). The two pol II bands, corresponding to the two phosphorylation states, have been described previously (47). The conversion of pol IIA to pol IIO in the presence of TFIIH requires ATP, correlates with conversion of the pol II largest subunit to a higher molecular weight form, and can be blocked by the kinase inhibitor H8 (47). To confirm that pol II was still present in lane 7, the CTD antibody was

FIG. 1. Isolated TFIIH does not interact with pol II ternary complexes. Standard electrophoretic mobility-shift assay utilized 300 ng of pol IIA, 150 ng of TFIIH, and 300 ng of recombinant CSB. Reactions were incubated in the presence or absence of 250 μM NTPs. Reactions were incubated for 40 min as described previously (47), then incubated with monoclonal antibodies against the pol II CTD (200 ng, lanes 5 and 8), the TFIIH subunit p62 (300 ng, lane 9), or the HA antibody as a negative control (500 ng, lane 10) for 5 min before loading. Reactions were resolved as described previously (65).
Recruitment of TFIIH to pol II-CSB Complexes

added, producing a supershift (lane 8). The results indicate that, although TFIIH can phosphorylate pol II, it does not interact with the complex. To confirm this, a monoclonal antibody directed against the p62 subunit of TFIIH (3C9, a kind gift of J.-M. Egly) was added to the complex (lane 9). No further shift was observed, although the 3C9 antibody did appear to block phosphorylation of the polymerase. Thus, under conditions in which pol II is known to be transcribing and stalling (47) (see below), TFIIH does not interact with the ternary complex. Similar results were found using labeled RNA-based gel mobility-shift assays (Fig. 3A).

The pol II-CSB-DNA-RNA Complex Interacts with an Activity Containing the Core TFIIH Subunits p62 and XBP/ERCC3

One hypothesis for the mechanism of TCR is that CSB recruits the TFIIH NER factor to the site of a DNA lesion, facilitating repair. This would explain why mutations in CSB, as well as certain mutations in TFIIH subunits, lead to CS (40). To address this, we added partially purified TFIIH to CSB-pol II-DNA-RNA complexes in both labeled RNA- and labeled DNA-based gel mobility-shift assays. We found that the CSB-containing complexes could interact with a p62- and XBP/ERCC3-containing factor present in partially purified TFIIH preparations.

Fig. 2 shows a labeled RNA-based gel mobility-shift assay in which partially purified TFIIH was added to stalled pol II-containing complexes in reactions containing purified recombinant CSB with a histidine tag at its NH2 terminus. In this assay, pol II is actively transcribing a labeled RNA from the DNA template, and the reactions are loaded onto a native 4.0% polyacrylamide gel containing 2 mM MgCl2. A pol II-A/O mixture produced two characteristic mobility shifts, due to the different phosphorylation states of the pol II CTD (47). CSB plus pol II produced a supershift and low mobility band (lane 3, brackets and single asterisk). Previous work has shown that the pol II ternary complex has the ability to recruit CSB in an ATP-dependent fashion (47). This interaction was verified using an anti-His6 monoclonal antibody (CLONTECH). The antibody interacts with pol II-CSB complexes (lane 4), but does not supershift pol II alone (lane 2). Monoclonal antibodies directed against hemagglutinin (HA; lane 5) and the p62 subunit of TFIIH (3C9; lane 6) were used as a negative control.

When partially purified TFIIH was added to a CSB-pol II ternary complex, a further supershift was observed (lane 7). Both the supershifted complex and the low mobility band were further shifted. TFIIH did not efficiently phosphorylate the pol II under these conditions, as very little protein was used and the time of incubation was relatively short. The supershift was slight, possibly due to the large molecular weight of the existing CSB-containing complex. Therefore, an interaction was confirmed using anti-TFIIHp62 antibodies, which generated a strong supershift (lane 8), while the HA antibody did not (lane 9).

Fig. 3A shows a similarly labeled RNA-based gel mobility-shift experiment in which partially purified TFIIH was added to stalled pol II-containing complexes in reactions containing CSB, CSA, or both. TFIIH alone did not produce a labeled complex, as TFIIH contained no transcription activity (lane 1). TFIIH added to pol II in the absence of CSB did not have a significant effect (lane 3). Monoclonal antibodies against the TFIIH core subunit XBP/ERCC3 (3G4/E5, a kind gift of Danny Reinberg) did not affect this complex, consistent with the interpretation that TFIIH is absent (lane 4). CSB plus pol II produced a typical supershift and low mobility band (lane 5, brackets and single asterisk). In this case, both phosphorylation states are visible in the supershifted band, and the ratio of phosphorylated to unphosphorylated pol II appeared to be the same as in the absence of CSB (lane 5, bracket). Taken together, this observation, the fact that pol II-CSB is shifted above the pol IIO mobility, and the fact that CSB is physically present in the complex, argues against a role for modification of pol II by CSB (for example by phosphorylation), although this remains a formal possibility.

Partially purified TFIIH added to the CSB-supershifted complex generated a further shift (lane 6). This pol II-CSB-DNA-RNA-TFIIH complex could be even further supershifted by anti-XBP antibodies (lane 7, double asterisk), but not by the nonspecific HA (12CA5) antibody (lane 8), indicating that this TFIIH core subunit is present in the complex. The pol II-CSB-DNA-RNA-TFIIH-XBP antibody complex is so large that it is shifted into the wells, even using a 4.0% polyacrylamide gel matrix with a relatively low (2.5%) cross-linking ratio.

Added CSA did not interact with the pol II complex under these conditions (lane 9), further demonstrating the specificity of the CSB interaction. This complex also did not interact significantly with TFIIH (lane 10). Consistent with this, both the XBP and HA antibodies did not shift the complex (lanes 11 and 12). When both CSA and CSB were included with pol II under transcribing conditions, similar results as with added CSB were observed. Once again, this complex could be specifically shifted with monoclonal antibodies against ERCC3, here generating a strong supershift (lane 15), while the HA antibody had no affect (lane 16).

We obtained similar results with labeled DNA-based gel mobility-shift assays (Fig. 3B), strongly suggesting that DNA and RNA are together in the same complex. As in the labeled
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Fig. 3. An XPB/ERCC3-containing factor can be recruited to a pol II-CSB-DNA/RNA complex. A, labeled RNA-based gel mobility-shift assay contained, where indicated, 75 ng of TFIIH, 200 ng of pol II, 300 ng of CSA, and 300 ng of CSB. After 20 min, 500 ng of the monoclonal anti-XBP or HA ascites antibody were added where indicated. Reactions were resolved using a gel matrix containing 2 mM MgCl₂. Electrophoresis was carried out under standard conditions (4 °C, 150 V, 3 h). Note that bands designated pol/CSB and pol/CSB/TFIIH also contain labeled DNA and unlabeled RNA.

B

DISCUSSION

Although pol II does not appear to directly interact with TFIIH in gel mobility-shift assays, the data support a model in which a molecular complex that contains the TFIIH subunits XPB/ERCC3 and p62 can interact with CSB, which can in turn interact with pol II in ternary complexes containing RNA (Fig. 3A). This model is depicted in Fig. 4 (right). It provides a possible biochemical basis for the phenomenon of facilitated NER in active genes, including the fact that cells derived from patients with mutations in CSB and certain subunits of TFIIH have a defect in this pathway.

CSA and CSB are implicated in the facilitated repair of active genes and linked to TFIIH both genetically and biochemically (reviewed in Friedberg (40)). Cells taken from Cockayne’s syndrome patients are defective in TCR (35–37). In addition, mutations in the XPB and XPD subunits of TFIIH and the TFIIH-associated repair factor XPG can also lead to symptoms of CS, and CSA and CSB have been shown, respectively, to interact with TFIIH and XPG in vitro. A recent report found that a species of TFIIH containing XPB/ERCC3 could be re-recruited (either directly or indirectly) from a HeLa nuclear extract by a fragment of the CSB protein (41). Therefore, it seemed reasonable that CSB could recognize DNA damage in active genes indirectly through pol II and recruit TFIIH or a TFIIH-like complex, facilitating DNA repair.

The observation that CSA had no effect on its own, and was not absolutely required for the interactions described here may be explained by that fact that CSB, and not CSA, appears to contain important enzymatic activities (41, 47, 50), and the finding that WD repeat-containing proteins are frequently regulatory in nature (51). TCR of oxidative base damage is only partially defective in CSA mutant cells, but fully defective in CSB and XPG/CS cells (37, 52). Two other possibilities are that the in vitro assay may be forced by the free addition of recombinant CSB, and the levels of protein found in vivo may necessitate the presence of CSA, or that the immunopurified pol II or baculovirus-purified CSB may have partially co-purified with endogenous CSA or an insect homologue.

The recruitment of TFIIH to stalled pol II complexes is reminiscent of TFIIH recruitment to transcription initiation complexes, and draws an interesting analogy with the pol II general transcription factor TFIIE (Fig. 4, left). CSB can interact with a stalled pol II elongation complex and recruit TFIIH. Similarly, TFIIE interacts with the pol II initiation complex and recruits TFIIH in reconstituted systems (53). TFIIE is also believed to modulate the enzymatic activities of TFIIH (8, 11, 54), and help stabilize an open DNA configuration (19). It will be interesting to determine whether or not the analogy can be extended to these phenomena as well.

Two studies have found that XPA, the main NER damage
recognition protein, has an affinity for TFIIH and recruits it to the site of damaged DNA (44, 45) (Fig. 4, center). XPC also associates with TFIIH and is required for NER (center). One possibility is that in TCR the repair mechanism operates in reverse, that is, first by recruitment of TFIIH to the lesion site indirectly through pol II and CSB. The TFIIH-XPA interaction may then bring XPA proximal to the lesion site and increase the likelihood of XPA damage recognition (Fig. 4, right). This idea is supported by the finding that CSB can also interact with XPA (41). The subsequent NER mechanism would then follow exactly as normal. Interestingly, XPC is not required for TCR (55). One intriguing possibility is that the CSB interaction surface on TFIIH is the same as or overlaps that for XPC, explaining the somewhat exclusive relationship between XPC and CSB.

This facilitated repair system would not replace, but be superimposed upon the normal NER pathway in which XPA recognizes the DNA lesion directly, without a targeting mechanism. Recent results (56) indicate that a damaged region of DNA covered by pol II does not undergo reduced rates of repair, but rather is repaired normally. Thus a TFIIH recruitment mechanism would increase the baseline levels of repair that are already in place. In the above study, added purified CSA and CSB had no effect. Perhaps a future system that includes the targeting step developed here may be applied toward recreating the entire coupling process with purified components, or toward the isolation of other components that are necessary for TCR. One advantage of this system is that only pol II need be added, so that the important roles of TFIIH can be addressed without a complicating requirement for transcription initiation.

An interesting possibility is that the forms of TFIIH recruited during transcription initiation, NER, and TCR are different. In support of this idea, a highly purified and transcriptionally active 8-subunit TFIIH complex (kindly provided by J. and R. Conanway) cannot be recruited to the CSB-containing complex in labeled RNA-based gel mobility-shift assays. Although free of other general transcription factors, pol II, and XPG, the most purified TFIIH preparations used in these experiments may still contain auxiliary factors that aid in its recruitment. It should be noted that more highly purified preparations that have lost the XPA protein can also generate a supershift (data not shown).

One or more factors not present in our assay system may also serve to further stabilize the aforementioned interactions or may act in later steps not modeled in our system, playing a key role in TCR in vivo. Candidate factors include TFIIE, TFIIF, XPG, and replication protein A. In addition the precise role of CSA remains to be deciphered. Furthermore, patients with manifestations of CS who do not fall into any “classic” complementation groups may harbor mutations in novel or unexpected genes encoding proteins that play an important role in this or related processes (57). Finally, because of the complexity of the CS phenotype, including neurological disorders and developmental abnormalities, and the heterogeneity of reports in the literature, it is likely that the CS proteins play important roles other than as recruitment factors for TFIIH. In this respect, it is interesting to note that preferential repair of oxidative damage can also be defective in CS (52), and that CSB interacts with the p53 protein in vitro (58). Recent data further indicate a potential role for the CSB protein in transcription elongation (59–61). Thus, the CS proteins may also be involved in targeting other repair factors, such as those involved in base excision repair, to active genes (62), in targeting p53 to respond to damage in active genes (63), and be involved in the transcription process directly.

Recently, it has been reported that simultaneous incubation of transcription and DNA repair templates with yeast extracts

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2 D. Reinberg, personal communication.

3 D. Tantin, unpublished observations.
leads to a partial loss of transcription efficiency. The effect is dependent upon the CSB protein, and can be reversed by the addition of yeast TFIIH (64). These results are fully consistent with those reported here.

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