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Different Forms of TFIIH for Transcription and DNA Repair: Holo-TFIIH and a Nucleotide Excision Repairosome

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Summary

Yeast TFIIH that is active in transcription can be dissociated into three components: a 5-subunit core, the *SSL2* gene product, and a complex of 47 kDa, 45 kDa, and 33 kDa polypeptides that possesses protein kinase activity directed towards the C-terminal repeat domain of RNA polymerase II. These three components can reconstitute fully functional TFIIH, and all three are required for transcription in vitro. By contrast, TFIIH that is highly active in nucleotide excision repair (NER) lacks the kinase complex and instead contains the products of all other genes known to be required for NER in yeast: *RAD1*, *RAD2*, *RAD4*, *RAD10*, and *RAD14*. This repairosome is not active in reconstituted transcription in vitro and is significantly more active than any of the constituent polypeptides in correcting defective repair in extracts from strains mutated in NER genes.

Introduction

RNA polymerase transcription factor IIH (TFIIH), also known as rat factor δ (Conaway and Conaway, 1989), yeast factor b (Feaver et al., 1991a), or human BTF2/TFIIH (Gerard et al., 1991; Flores et al., 1992), is essential for initiation at RNA polymerase II promoters in vitro. In yeast (human), TFIIH contains products of the *SSL2* (*XPB*) (Feaver et al., 1993; Bardwell et al., 1994a; Schaeffer et al., 1993), *RAD3* (*XPD*) (Feaver et al., 1993; Schaeffer et al., 1994; Drapkin et al., 1994a), *TFB1* (*p62*) (Gileadi et al., 1992; Fischer et al., 1992), and *SSL1* (*p44*) (Feaver et al., 1993; Humbert et al., 1994) genes, which are required for nucleotide excision repair (NER) of DNA. TFIIH appears to function in NER since it can complement deficiencies in the repair of extracts from yeast strains or human cell lines mutant in NER genes (Wang et al., 1994; Schaeffer et al., 1994; van Vuuren et al., 1994; Drapkin et al., 1994b; Wang et al., submitted). The dual roles of TFIIH may underlie the coupling of NER and transcription as revealed by the preferential repair of DNA damage in the transcribed strand of active genes (Bohr et al., 1985; Mellon et al., 1987; Smerdon and Thomas, 1990).

In previous work, yeast TFIIH was isolated both as a 5-subunit core complex (core TFIIH), including the repair proteins RAD3, TFB1, and SSL1 (Feaver et al., 1993; Svejstrup et al., 1994), and as a 9-subunit holoenzyme (holo-TFIIH), comprising the core plus SSL2 protein and three additional polypeptides of 47 kDa, 45 kDa, and 33 kDa size (Svejstrup et al., 1994). Core TFIIH and holo-TFIIH can be distinguished by three types of functional assay, one measuring protein kinase activity directed towards the C-terminal repeat domain (CTD) of RNA polymerase II (Feaver et al., 1991b) and the other assays measuring promoter-specific transcription in heat-treated nuclear extract (HNE) (Feaver et al., 1991a) or in a system reconstituted from pure yeast TFIIB, TFIIE, TFIIF, TATA-binding protein, and RNA. Holo-TFIIH is active in all three assays. Core TFIIH, on the other hand, is active in HNE transcription but fails to support either CTD phosphorylation or transcription in the reconstituted system. Evidently, both CTD kinase and reconstituted transcription activities reside in the additional polypeptides present in holo-TFIIH, or alternatively, these activities require interaction of the core with the additional polypeptides. Function of the core protein in the HNE assay suggests that the additional polypeptides of holo-TFIIH survive heat treatment and associate reversibly with the core.

The evidence for multiple forms of TFIIH and the reported interaction of the factor with other DNA repair proteins (Bardwell et al., 1994b) prompted us to pursue structure–function relationships of TFIIH. The results necessitate a reevaluation of proposals for the apparent involvement of TFIIH in both repair and transcription (Feaver et al., 1993; Bootsma and Hoeijmakers, 1993; Cleaver, 1994; Drapkin et al., 1994a; Hanawalt et al., 1994). We find that TFIIH competent for NER differs markedly from the protein active in transcription. Our results also shed light on the mechanisms of repair and repair–transcription coupling in eukaryotes.

Results

Dissociation and Reconstitution of Holo-TFIIH: CTD Kinase Complex

The subunit-activity relationships in holo-TFIIH were investigated by dissociation and reconstitution experiments. To this end, nearly homogeneous holo-TFIIH, bearing a 6-histidine tag at the C-terminus of the TFB1 subunit, was adsorbed to Ni^{2+}–NTA–agarose and was washed with solutions of increasing salt concentration (but not so concentrated as to release core TFIIH from the column). SSL2 protein was retained in holo-TFIIH under all conditions tested, but the 47 kDa, 45 kDa, and 33 kDa polypeptides were partially eluted at concentrations of greater than ~400 mM potassium acetate (Figure 1A). The RAD3 subunit of core TFIIH also appeared to dissociate, though it did not precisely coelute with the three smaller polypep-

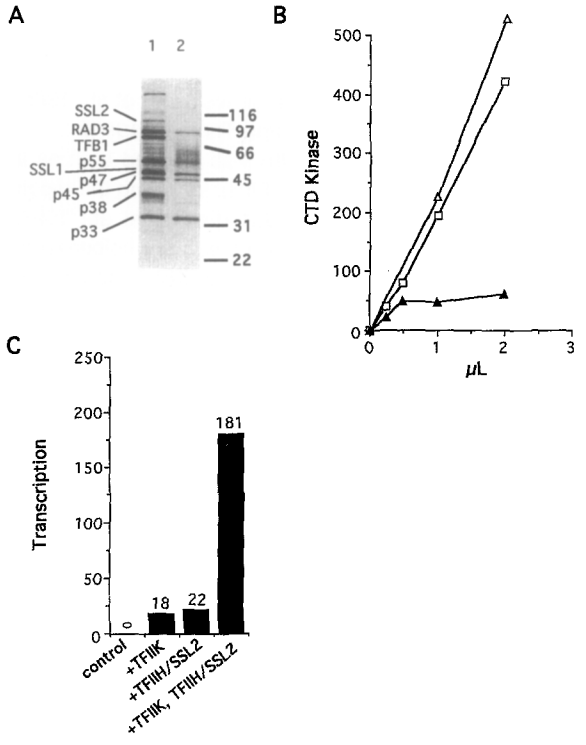

Figure 1. Dissociation of TFIIK, the CTD Kinase, from Holo-TFIIH and Its Requirement for Transcription

(A) Purified holo-TFIIH (lane 1) was bound to Ni^{2+}–NTA–agarose and washed with high salt buffer, dissociating TFIIK (lane 2). The five core TFIIH subunits, SSL2, and three TFIIK subunits (designated according to apparent molecular weight) are indicated at the left. Molecular weights of protein standards (Bio-Rad) are indicated in kilodaltons at the right. The staining at and immediately above 55 kDa in lane 2 is an artifact owing to extended silver staining. In other similar experiments, p55 did not seem to dissociate from holo-TFIIH.

(B) CTD kinase assays with holo-TFIIH (open triangles; from [A], lane 1), TFIIK (open squares; from [A], lane 2), and core TFIIH–SSL2 (Svejstrup et al., 1994) (closed triangles).

(C) Reconstituted transcription assays performed without any component of TFIIH (control), with TFIIK alone (from A, lane 2), with coreTFIIH–SSL2 (Svejstrup et al., 1994) (designated TFIIH/SSL2), or with both TFIIK and core TFIIH–SSL2. Incorporation in specific transcripts is indicated in arbitrary units above the bars.

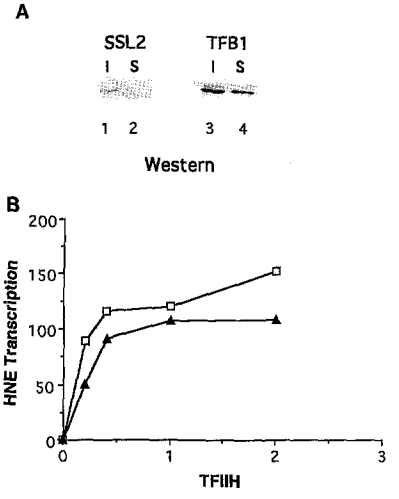

Figure 2. SSL2-Depleted TFIIH Retains Transcriptional Activity in HNE

(A) Load (I) and supernatant from immunodepletion (S) with anti-SSL2 antibodies analyzed by immunoblotting with anti-SSL2 (lanes 1 and 2) or anti-TFB1 (lanes 3 and 4) antisera.

(B) HNE transcriptional activities of immunodepletion load (open squares) and supernatant (closed triangles).

tides (Figure 1A; data not shown). Similar dissociation has been reported for the homologous xeroderma pigmentosum D (XPD) polypeptide in the human protein BTF2/TFIIH (Schaeffer et al., 1993, 1994).

The 400 mM potassium acetate eluate from Ni-bound holo-TFIIH exhibited CTD kinase activity that was virtually indistinguishable from that of the starting holo-TFIIH (Figure 1B; data not shown). In contrast, purified core TFIIH was essentially inactive. CTD kinase activity thus resides in the 47 kDa, 45 kDa, and 33 kDa polypeptides, which appear to form a complex since they copurified through an additional chromatographic step (data not shown). Neither this kinase nor the residual core TFIIH–SSL2 complex was alone sufficient for transcription in the reconstituted system, whereas together they supported a reaction comparable to that observed with holo-TFIIH (Figure 1C). We conclude that both kinase and core TFIIH–SSL2 complexes are essential for RNA polymerase II transcription. The kinase may be viewed as a distinct, essential, general transcription factor, which we refer to as TFIIK.

Dissociation and Reconstitution of Holo-TFIIH: SSL2 Protein

A similar analysis was performed to distinguish the contributions of core TFIIH and SSL2 to transcription. We previously described a highly purified TFIIH preparation containing a barely detectable amount of SSL2 protein, which is active in reconstituted transcription (Feaver et al., 1993; Svejstrup et al., 1994). To determine whether this trace quantity of SSL2 is essential for transcription, it was removed by immunodepletion with anti-SSL2 antibodies. No SSL2 remained, as judged from immunoblotting, while the level of core TFIIH, revealed by that of the TFB1 subunit, was only slightly diminished (Figure 2A). Depletion of SSL2 had no significant effect on transcriptional activity in the HNE assay (Figure 2B).

In transcription reactions reconstituted with pure transcription proteins, SSL2 depletion completely abolished activity (Figure 3A). SSL2 depletion also abolished CTD kinase activity (Figure 3B), which likely reflects holo-TFIIH depletion, SSL2–TFIIK interaction, or the recognition of epitopes in TFIIK by the polyclonal anti-SSL2 antibody. These results are in keeping with our previous conclusion that the transcriptional activity of the highly purified core TFIIH preparation was due to the presence of a small amount of holo-TFIIH (Svejstrup et al., 1994).

Direct evidence for the requirement for SSL2 protein in transcription came from adding the purified protein back to SSL2-depleted TFIIH (core TFIIH) in reconstituted transcription assays. SSL2 was modified with a 6-histidine tag, expressed in yeast, and was purified by chromatography

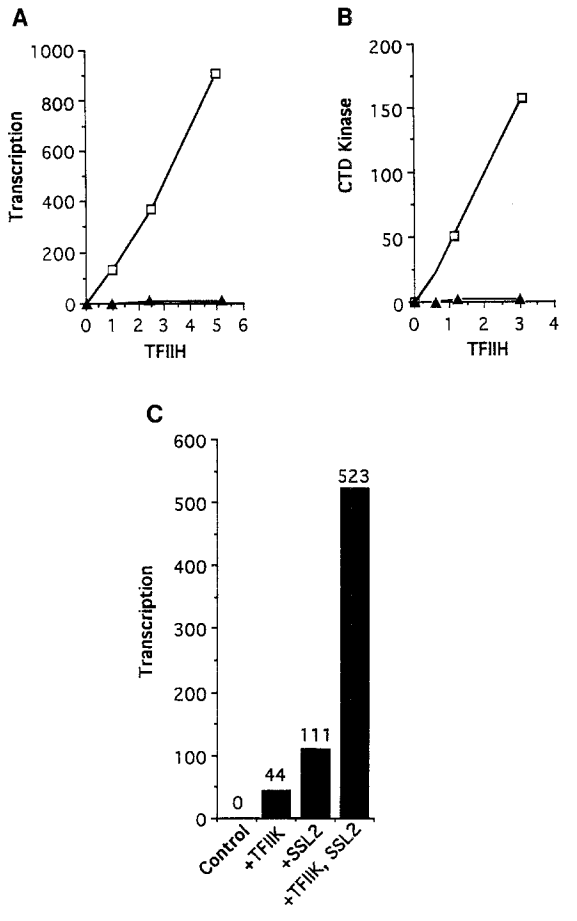

Figure 3. SSL2 Is Required for Transcription Reconstituted with Pure Proteins

(A) Reconstituted transcriptional activities of immunodepletion load (open squares) and supernatant (closed triangles) from Figure 2.

(B) CTD kinase activities of immunodepletion load (open squares) and supernatant (closed triangles).

(C) Reconstitution of holo-TFIIH from core, TFIIK, and SSL2. Reconstituted transcription activities of core TFIIH alone (immunodepletion supernatant, designated Control), core TFIIH and TFIIK (from Figure 1A, lane 2; designated +TFIIK), core TFIIH and SSL2 (+SSL2), and all three components (+TFIIK, SSL2). Results are presented as in Figure 1C, with incorporation in specific transcripts indicated in arbitrary units above the bars.

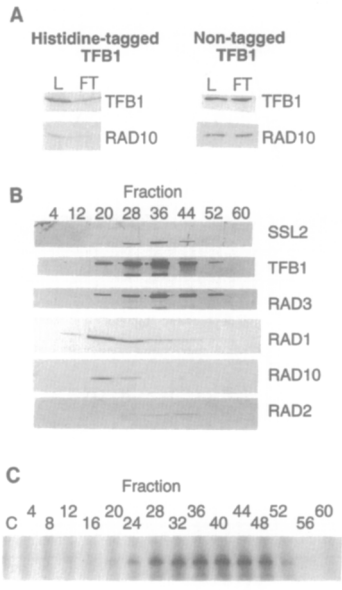

Figure 4. Fractionation of the NER Repairosome

Whole-cell extracts from yeast strains expressing 6-histidine-tagged TFB1 or nontagged TFB1 were fractionated on Bio-Rex 70, phosphocellulose, and Ni^{2+}–NTA–agarose as described (Svejstrup et al., 1994), with the exceptions noted in Experimental Procedures.

(A) Load (L) and flowthrough (FT) from Ni–agarose (20 μg) were analyzed by immunoblotting with anti-TFB1 and anti-RAD1 antisera.

(B) Ni–agarose fractions (4 μl for RAD3, SSL2, and TFB1; 20 μl for RAD1, RAD2, and RAD10) were analyzed by immunoblotting with the antisera indicated.

(C) HNE transcription assays of Ni–agarose fractions (0.1 μl). Transcripts were resolved by polyacrylamide gel electrophoresis in a 7% denaturing gel, followed by autoradiography.

on Ni–agarose, Bio-Rex 70, Ni–agarose, and Mono Q. The peak Mono Q fraction was devoid of HNE transcriptional activity and of CTD kinase activity (data not shown), indicating the absence of contaminating core TFIIH and TFIIK. Purified SSL2 protein, TFIIK, and SSL2-depleted core TFIIH were tested in all combinations for transcriptional activity in the reconstituted system. Only when all components were added in combination was appreciable transcription obtained (Figure 3C; data not shown). We conclude that all three components of holo-TFIIH (core TFIIH, TFIIK, and SSL2 protein) are essential for RNA polymerase II transcription.

DNA Repairosome

In addition to its association with TFIIK and SSL2, highly purified core TFIIH interacts with RAD2 and RAD4 proteins (Bardwell et al., 1994b), which are essential for NER but are not required for transcription. In view of the reversible association and apparent loss of TFIIK and SSL2 from holo-TFIIH during isolation, we investigated whether a larger complex containing additional RAD proteins might also be disrupted during isolation. To preserve such a complex, TFIIH bearing a 6-histidine tag on the TFB1 subunit was purified as described (Svejstrup et al., 1994), except with the maintenance of a higher protein concentration prior to the Ni–agarose step and with lower ionic strengths for washing bound protein in all chromatographic steps. To detect a complex with additional RAD proteins, the load and flowthrough of the Ni–agarose column were analyzed for the presence of TFB1 and RAD10 proteins by immunoblotting. Both proteins were depleted to a comparable extent in the flowthrough fraction, whereas no depletion was observed in a preparation from a yeast strain expressing wild-type (untagged) TFB1 (Figure 4A). This evidence for a TFB1–RAD10 complex was particularly noteworthy in view of the lack of interaction between core TFIIH and the RAD1–RAD10 heterodimer in immunoprecipitation experiments (Feaver et al., 1993). In all likelihood, RAD1–RAD10 interacts indirectly with core TFIIH through contact with other TFIIH-associated proteins.

Additional components of the TFB1–RAD10 complex

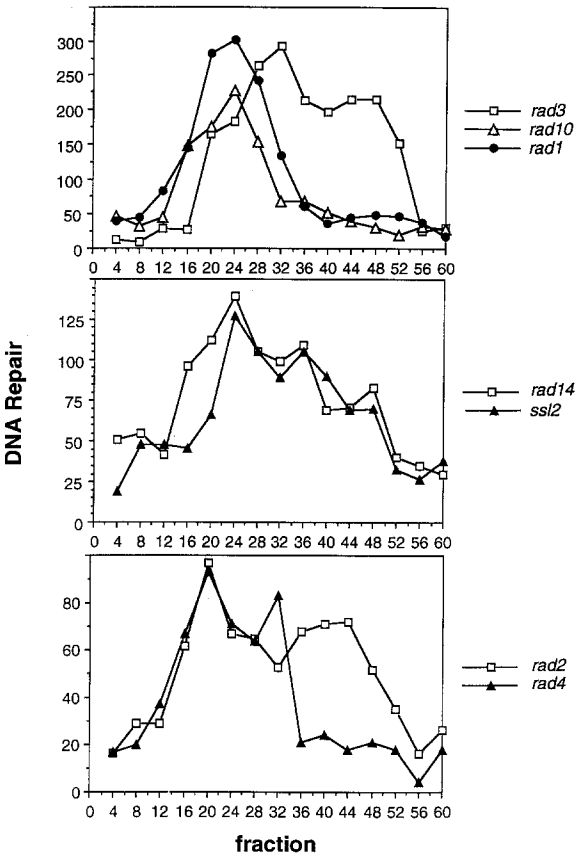

Figure 5. Coelution of In Vitro Repair Activities from Ni–Agarose

NER assays of Ni–agarose fractions with extracts from strains mutant in the NER genes indicated. Products of NER reactions were resolved in a 1% agarose gel, followed by autoradiography. Incorporation of dCMP in damaged DNA is plotted on the ordinate.

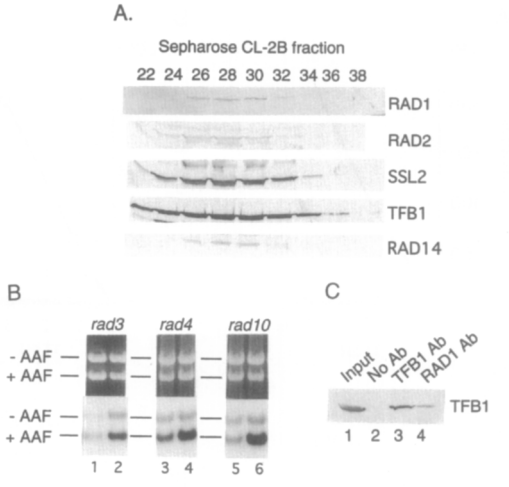

Figure 6. Characterization of the NER Repairosome

(A) Comigration of NER proteins (indicated on the right) on Sepharose CL-2B, as revealed by immunoblotting.

(B) NER activities of RAD3, RAD4, and RAD10 in Sepharose CL-2B peak. NER in extracts of the mutant strains indicated was assayed without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) the addition of Sepharose CL-2B fraction 29.

(C) Immunoprecipitation of TFB1 from Sepharose CL-2B fraction 29 (Input) with anti-TFB1 (TFB1 Ab), anti-RAD1 (RAD1 Ab), or no (No Ab) antibodies, as revealed by immunoblotting with anti-TFB1 antiserum.

were revealed by immunoblotting of fractions eluted from the Ni–agarose column (Figure 4B). RAD3, TFB1, and SSL2 proteins of the core TFIIH–SSL2 complex were broadly distributed in fractions 28–44, peaking in fraction 36; RAD1 and RAD10 proteins eluted earlier, in fractions 20–28, peaking in fraction 20; and RAD2 protein eluted later than the core TFIIH–SSL2 complex, in fractions 36–44. The profile of transcriptional activity in the HNE assay (Figure 4C) was similar to that of the core TFIIH proteins. Profiles of NER activity determined for each protein by complementation of the corresponding repair-deficient mutant extract, however, deviated significantly in some cases from the protein distribution (Figure 5). NER activities of RAD3, SSL2, and RAD2 proteins were displaced towards earlier eluting fractions. This behavior was most pronounced for RAD2, the NER activity of which peaked in fraction 20, although the protein was undetectable by immunoblotting in this fraction. All seven NER activities that were tested peaked in similar early eluting fractions, especially RAD1 and RAD10, which were narrowly distributed in fractions 16–32. We surmise that Ni–agarose fractions 16–32 contained a complex of all known proteins essential for NER (TFB1, SSL1, SSL2, RAD1, RAD2, RAD3, RAD4, RAD10, RAD14), referred to as a "repairosome", the exceptional activity of which derives from proximity relationships of proteins in the complex.

Three further lines of evidence support the existence of such a repairosome. First, putative repairosome fractions from Ni–agarose were filtered through Sepharose CL-2B. All NER proteins that were analyzed comigrated, as evidenced by immunoblotting (Figure 6A) and repair assays (Figure 6B), which is consistent with their association in a large complex. Second, anti-RAD1 antibodies coprecipitated TFB1 (Figure 6C) and RAD14 (data not shown) and, conversely, anti-TFB1 antibodies coprecipitated RAD1 and RAD14 (data not shown). These proteins were evidently in the same complex rather than simply comigrating on Sepharose CL-2B. A third line of evidence came from the comparison of core TFIIH to the putative repairosome (Sepharose CL-2B fraction 29) by filtration through Bio-Sil SEC400 (data not shown). Core TFIIH migrated as an ~250 kDa protein relative to marker proteins, in keeping with previous findings (Feaver et al., 1991a) and as expected for the sum of the constituent polypeptides. On the other hand, the repairosome from Sepharose CL-2B fraction 29 migrated as a larger complex with an apparent mass between 700 kDa and 1000 kDa, which is consistent with an assembly of all known essential NER proteins, the total mass of which is ~850 kDa.

Functional Distinctions between Holo-TFIIH and the NER Repairosome

The three functional assays described above for holo-TFIIH, which measure CTD kinase activity and transcription activities in HNE and reconstituted systems, were applied to the repairosome. No CTD or other kinase activity

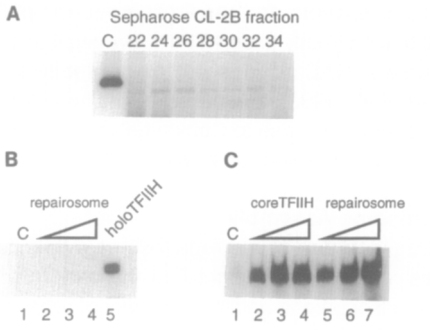

Figure 7. Functional Comparisons of Holo-TFIIH, Core TFIIH, and the NER Repairosome

(A) CTD kinase assays of holo-TFIIH (control, designated C) and Sepharose CL-2B fractions (1 μl). Phosphorylated proteins were resolved by SDS–polyacrylamide gel electrophoresis in a 7.5% gel, followed by autoradiography. The single labeled band produced by holo-TFIIH corresponds to the phosphorylated form of the largest subunit of RNA polymerase II, RPB1.

(B) Reconstituted transcription assays without the addition of TFIIH (control, designated C), with Sepharose CL-2B fraction 29 (1 μl, 2 μl, and 5 μl in lanes 2, 3, and 4, respectively), or with holo-TFIIH.

(C) HNE transcription assays without the addition of TFIIH (control, designated C), with Sepharose CL-2B fraction 29 (1 μl, 2 μl, and 5 μl in lanes 2, 3, and 4, respectively), or with core TFIIH (Feaver et al., 1993; Svejstrup et al., 1994) (0.1 μl, 0.2 μl, and 0.5 μl in lanes 5, 6, and 7, respectively). Holoenzyme polymerase II (200 ng) (Kim et al., 1994) was used in place of core polymerase, and TFIIF was therefore omitted in the reconstituted transcription reactions. The amount of holo-TFIIH (from Figure 1A) used as control (from [A] and [B]) was one-fifth that of the TFIIH in Sepharose CL-2B fraction 29, as judged from quantiative Western blots of the TFB1 subunit.

was detected comigrating with the repairosome on Sepharose CL-2B (Figure 7A), although kinase activity with other protein targets was detected in several fractions (Figure 7A; data not shown). Moreover, the CTD kinase was absent from the fractions, as shown by immunoblotting with antibodies directed against 1 of the 3 subunits of TFIIK (data not shown). Consistent with the lack of kinase activity and the requirement for TFIIK in the reconstituted transcription system, the repairosome was also inert in reconstituted transcription (Figure 7B). In contrast, repairosome fractions were functional in the HNE transcriptional assay (Figure 7C), possibly reflecting the exchange of TFIIK in the extract for NER proteins of the repairosome.

Discussion

Implications for Transcription and NER

The resolution of holo-TFIIH into core, CTD kinase, and SSL2 proteins and the reconstitution of these components in functional form has 2-fold significance. First, the CTD kinase function of TFIIH is identified with a discrete complex that is essential for transcription, termed TFIIK. The subunit composition and chromatographic behavior of TFIIK distinguish it from previously described CTD kinases (Lee and Greenleaf, 1989; Cisek and Corden, 1989). Moreover, recent results show that the 33 kDa subunit of TFIIK is identical to KIN28 (Feaver et al., 1994b), a member of the $p34^{CDC2/CDC28}$ family of protein kinases (Simon et al., 1986). Second, our experiments provide direct biochemical evidence that SSL2 protein is required for transcription, confirming indications from previous studies of this gene product and its human homolog, XPB (Schaeffer et al., 1993; van Vuuren et al., 1994; Drapkin et al., 1994a; Svejstrup et al., 1994; Qui et al., 1993; Guzder et al., 1994).

CTD phosphorylation is dispensable for transcription in fully defined transcription systems (Serizawa et al., 1993; Li and Kornberg, 1994). It is therefore somewhat unexpected that TFIIK is required for transcription in the reconstituted system. Possibly the subunits of TFIIK supply other important catalytic or regulatory activities or play a structural role in the assembly and maintenance of holo-TFIIH and the transcription initiation complex.

The existence of a repairosome, a complex of all known proteins that are indispensable for NER in yeast, which is assembled in the absence of exposure to DNA-damaging agents, indicates a fundamental mechanistic difference from prokaryotes in which NER appears to proceed by the sequential assembly and action of the UvrA, UvrB, and UvrC proteins at sites of damage (reviewed by Sancar and Tang, 1993). The repairosome defined in our studies contains proteins required for many of the biochemical functions intrinsic to NER, including RAD3 (Naegeli et al., 1992) and RAD14 (Guzder et al., 1993) for damage-specific recognition, RAD3 (Sung et al., 1987) and SSL2 (Guzder et al., 1994) for bidirectional helicase activity, and RAD1–RAD10 (Tomkinson et al., 1993) and RAD2 (Habraken et al., 1993) for two incision activities. We have not detected NER in assays with the repairosome alone (Z. W. and E. C. F., unpublished data). This presumably reflects the absence of additional components that are required for the process, such as DNA polymerase, DNA ligase, and various polymerase accessory factors that are known to influence NER in human extracts (Coverly et al., 1992; Shivji et al., 1992; Nichols and Sancar, 1992).

Repair-Transcription Coupling

Holo-TFIIH and the NE repairosome share a common core TFIIH–SSL2 complex, which appears (in light of the following) to associate reversibly with either TFIIK or NER proteins. First, holo-TFIIH was as active as the core complex in correcting defective NER in a *rad3* mutant extract (data not shown), which is indicative of the dissociation of TFIIK and its replacement by NER proteins. Second, the repairosome was as active as core TFIIH in HNE transcription (Figure 7C), which is indicative of the replacement of NER proteins by TFIIK. Third, the dissociation of TFIIK from holo-TFIIH and its reconstitution with core complex have been directly demonstrated (Figures 1–3). Interchange between holo-TFIIH and the repairosome could underlie repair–transcription coupling. For example, when associated with RNA polymerase at a promoter, core TFIIH might bind TFIIK most avidly, whereas in the presence of DNA damage, the core might undergo a conformational change that results in the release of TFIIK and the recruitment of further NER proteins. The affinity of core TFIIH for RNA polymerase that is paused at sites of DNA damage, perhaps in conjunction with transcription-coupling repair proteins such as RAD26 (yeast homolog of human ERCC6

[van Gool et al., 1994]), would assure efficient coupling. Altogether different possibilities merit consideration, such as the formation of a larger complex containing both TFIIK and NER proteins of the repairosome. The stability of such a complex might require RNA polymerase stalled at a site of damage or transcription-coupling repair proteins, accounting for the absence of a larger complex in our experiments. Alternatively, such a complex might not be purified in sufficient quantities for detection because it is even more fragile than holo-TFIIH and the repairosome. Roy et al. (1994) recently reported that antibodies against MO15, the human counterpart of the KIN28 catalytic subunit in TFIIK, inhibit DNA repair in vivo. Further studies will show whether MO15 antibody in these studies simply served to deplete human holo-TFIIH and prevent its conversion to a repairosome or whether a repairosome can actually be assembled around holo-TFIIH.

Interchange between holo-TFIIH and NER repairosome would also afford an indirect mechanism of repair–transcription coupling. Extensive DNA damage leading to the induction of RAD2 protein (reviewed by Friedberg et al., 1991) may result in assembly of the repairosome at the expense of holo-TFIIH. Under conditions of holo-TFIIH limitation, the frequency of transcription initiation would then be diminished.

Correspondence between Biochemical and Genetic Findings

The distinction made here between core and associated proteins of the repairosome on biochemical grounds is remarkably concordant with the genetic requirements for cell viability and NER in yeast. The *RAD3*, *TFB1*, *SSL1*, and *SSL2* genes are all essential for viability, presumably because their protein products are present in the core–SSL2 complex, which is required for transcription. The *RAD1*, *RAD2*, *RAD4*, *RAD10*, and *RAD14* genes, on the other hand, are not essential for viability (reviewed by Friedberg et al., 1991), which is consistent with their absence from the transcriptional form of TFIIH and their presence only in the alternative form of the factor involved in NER. The dichotomy of TFIIH structure is thus reflected in TFIIH genetics.

With the exception, at present, of RAD10, all the yeast proteins discussed here have human homologs that are implicated in the hereditary disease XP (reviewed by Tanaka and Wood, 1994). In addition, components of (core) BTF2/TFIIH are implicated in Cockayne's syndrome associated with XP and in trichothiodystrophy (Schaeffer et al., 1993, 1994; Drapkin et al., 1994a), both of which can manifest NER defects. Cockayne's syndrome and trichothiodystrophy exhibit extraordinary phenotypic complexity, including diverse developmental and neurological defects, which might be attributed to deficiencies in components of holo-TFIIH directly involved in transcription, such as XPB (SSL2) and XPD (RAD3). However, Cockayne's syndrome can also result from defects in the *XPG* gene (Tanaka and Wood, 1994), the homolog of *RAD2*, the product of which is not included in holo-TFIIH but only in the repairosome. Mutations in *RAD2* might affect transcription indirectly, for example, by perturbing interconversion between holoenzyme and repairosome forms of TFIIH, as RAD2 binds directly to coreTFIIH (Bardwell et al., 1994b). Conversely, RAD14 does not bind directly to the core (Feaver et al., 1993), and defects in its homolog, XPA, do not appear to give rise to Cockayne's syndrome or trichothiodystrophy (Tanaka and Wood, 1994).

Repairosome Assembly

The fragile nature of the repairosome evidenced by our studies may provide insight into the pattern of protein–protein associations and the pathway of repairosome assembly. Secondary peaks of all NER proteins (except RAD1–RAD10), eluting from Ni–agarose later than the fully assembled repairosome (Figure 5), point to the existence of subcomplexes of the repairosome. The presence of RAD1–RAD10 in only a single peak containing the complete repairosome suggests that these proteins are recruited in the last step of complex assembly. Like RAD1–RAD10, RAD14 fails to bind to isolated core TFIIH, but it might bridge the association of RAD1–RAD10 with the other direct core-binding proteins. Indeed, in the human system, interactions have been identified between XPA, ERCC4 (XPF), and ERCC1 (Li et al., 1994; Park and Sancar, 1994), the homologs of RAD14, RAD1, and RAD10, respectively. The recent purification of a complex of XPC and HHR23 (Masutani et al., 1994), human homologs of RAD4 and RAD23, suggests that RAD23 may also be incorporated in the repairosome. RAD7 and RAD16, which (like RAD23) are involved in but not essential for NER in yeast (Friedberg et al., 1991), may be associated with the repairosome as well.

Experimental Procedures

Purification of Holo-TFIIH and TFIIK

Holo-TFIIH purification and all buffers and reagents were as described (Svejstrup et al., 1994). For dissociation of TFIIK from holo-TFIIH, a Mono Q HR5/5 fraction (100 μl) containing holo-TFIIH (10 μg) was utilized. The fraction was not contaminated with any other basal transcription factor (see Figure 5B in Kim et al., 1994; J. Q. S., unpublished data). After dialyzing against buffer L-0.15 for 2 hr, the potassium acetate concentration of the fraction was adjusted to 150 mM by dilution with buffer L-0, and binding was performed in batch for 4 hr with 30 μl of Ni^{2+}–NTA–agarose equilibrated in buffer L-0.15. The Ni–agarose beads were collected by centrifugation and washed twice with 75 μl each of buffers L-0.15, L-0.4, L-0.8, L-1.2, L-1.6, and L-0.1 containing 100 mM imidazole. The load and 400 mM potassium acetate eluate are shown (Figure 1A, lanes 1 and 2, respectively).

Purification of SSL2 Protein

For the preparation of SSL2 protein, the *SSL2* gene was modified to append a 6-histidine tag at the N-terminus, cloned into YEP435 (Ma et al., 1987) and overexpressed in yeast by transcription from the *GAL1* promoter (Chasman and Kornberg, 1990). Whole-cell extract (210 mg in 80 ml) was prepared as described (Sayre et al., 1992) and precipitated by addition of ammonium sulfate to a final concentration of 80% with stirring for 30 min. The precipitate was collected by centrifugation in a Beckman JA-20 rotor at 16,000 rpm for 30 min, dissolved in 12 ml of buffer I-0, and bound in batch for 2 hr to 1 ml of Ni^{2+}–NTA–agarose equilibrated in buffer I-1.0. The Ni–agarose beads were washed in a column (0.7 cm in diameter) with 10 ml of buffer I-1.0 and 10 ml of buffer I-0.15 containing 20 mM imidazole. Bound protein was eluted with buffer I-0.15 containing 200 mM imidazole and was applied to a 1 ml column of Bio-Rex 70 equilibrated with buffer A-0.15. The column was washed with 5 ml of buffer A-0.15 and was eluted with buffers I-0.4, I-0.8, and I-1.2. SSL2 eluting from this and subsequent columns

was detected by Western blotting. The buffer I-0.8 eluate (1.5 ml) was bound in batch for 2 hr to 50 μl of Ni–agarose equilibrated in buffer I-1.0, and the Ni–agarose beads were collected by centrifugation, washed in batch with buffer I-1.0 and buffer I-1.0 containing 20 mM imidazole, and eluted with buffer I-0.2 containing 200 mM imidazole. The eluate (400 μl) was applied at 0.3 ml/min to a 1 ml Resource Q column (Pharmacia) equilibrated in buffer B-0.2. The column was washed with 2 ml of buffer B-0.2 and eluted with a gradient of 0.2–1.0 M buffer B. SSL2 eluted between 475 mM and 525 mM potassium acetate and was not contaminated with TFIIH or kinase activity detectable in HNE transcription and CTD kinase assays.

Purification of NE Repairosome

Repairosome was purified from whole-cell extracts from yeast strains expressing 6-histidine-tagged TFB1 (Svejstrup et al., 1994) or non-tagged TFB1 (S14–0; Song et al., 1990) by fractionation on Bio-Rex 70, phosphocellulose, and Ni^{2+}–NTA–agarose, as described for holo-TFIIH (Svejstrup et al., 1994), with the following exceptions: whole-cell extract (160 g) was applied to 4 l of Bio-Rex 70 and was washed with buffers A-0.2 and A-0.3 before elution with buffer A-0.65. After dialysis, the 0.65 M eluate (11.5 g) was loaded onto 1 l of phosphocellulose and was washed with buffers A-0.2 and A-0.3 before elution with buffer A-0.6. The 0.6 M eluate (4 g) was precipitated by the addition of ammonium sulfate to 75% of saturation with stirring and centrifugation for 30 min at 30,000 rpm in a Beckman Ti42 rotor. The pellet (3.5 g) was dissolved in 90 ml of buffer J-0, dialyzed against buffer J-0 to the conductivity of buffer J-0.3, bound in batch to 5 ml of Ni–agarose, and eluted with a gradient of 10–100 mM imidazole in buffer J or by a 20 mM step elution. The Ni–agarose eluate in each case was approximately 3000-fold enriched in TFIIH compared with whole-cell extract, estimated from quantitative Western blots of the TFB1 subunit. Fractions from Ni–agarose (20 mM imidazole eluate) was applied at 4 ml/hr to Sepharose CL-2B (Pharmacia, 450 mm × 10 mm) equlibrated in 20 mM HEPES (pH 7.6), 20% glycerol, 300 mM potassium acetate, 0.01% Nonidet P-40, 1 mM β-mercaptoethanol, and protease inhibitors. For size determination of the repairosome, 200 μl of Sepharose CL-2B fraction 29 was subjected to Bio-Sil SEC400 HPLC (Bio-Rad) at 0.5 ml/min in the Sepharose CL-2B buffer. The elution profile of the repairosome was compared with that of size markers (high molecular weight markers from Bio-Rad) and with that of core TFIIH (Feaver et al., 1993; Svejstrup et al., 1994) by immunoblotting with anti-TFB1 antiserum.

In Vitro Assays: Transcription, CTD Phosphorylation, and DNA Repair

CTD phosphorylation, HNE transcription, and reconstituted transcription were performed, and incorporation of ^{32}P in specific transcripts or in RPB1 (CTD phosphorylation) was quantified as described (Svejstrup et al., 1994; Feaver et al., 1991a, 1991b). All numerical values were normalized to the amounts of TFIIH in compared fractions, estimated from quantitative Western blots of the TFB1 subunit.

In vitro repair using repair-deficient mutant yeast strains was done as described (Wang et al., 1993, 1994).

Immunological Methods

Immunodepletion of core TFIIH–SSL2 (2 μg in 100 μl) was performed three times with anti-SSL2 antiserum (75 μl) bound to 50% protein A–Sepharose beads (50 μl), according to Cairns et al. (1994). Likewise, immunoprecipitation of Sepharose CL-2B fraction 29 (100 μg in 100 μl) entailed three consecutive treatments with 20 μl of the relevant 50% antibody protein A–Sepharose beads. The beads were washed five times with 100 μl of PBS containing 5% PEG and 100 mM potassium acetate, and bound proteins were analyzed by immunoblotting.

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