Yeast RAD3 protein binds directly to both SSL2 and SSL1 proteins: Implications for the structure and function of transcription/repair factor b

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ABSTRACT The *RAD3* and *SSL2* gene products are essential proteins that are also required for the nucleotide excision repair pathway. We have recently demonstrated that the *RAD3* gene product along with the *SSL1* and *TFB1* gene products are subunits of RNA polymerase II basal transcription factor b. Additionally, the *SSL2* gene product physically interacts with purified factor b. Here we combine an *in vitro* immunoprecipitation assay and an *in vivo* genetic assay to demonstrate a series of pairwise protein–protein interactions involving these components. *RAD3* protein binds directly to both *SSL2* protein and *SSL1* protein *in vitro*. *SSL1* protein interacts with itself and with *RAD3* and *TFB1* proteins in living yeast cells. An N-terminal, possibly noncatalytic, domain of *SSL2* protein is sufficient for the factor b–*SSL2* interaction, and a product of a DNA repair-defective allele of *SSL2* is not defective in binding to factor b. We present genetic evidence suggesting that the DNA-repair function of *SSL2* protein is not dependent on its essential function.

DNA repair, transcription, replication, and recombination are accomplished by complex biochemical pathways, some of which intersect and overlap. The yeast *Saccharomyces cerevisiae* has been investigated in our laboratories as a eukaryotic model for RNA polymerase II transcription and nucleotide excision repair (NER) and for the coupling of these two processes. More than 10 yeast genes involved in NER have been cloned and sequenced (1). Among these genes *RAD3* and *SSL2* (also called *RAD25*) encode a known and a presumed DNA helicase (2–5) and are exceptional in that they are also essential for cell viability in the absence of DNA damage (4–6). The human homologs of the yeast *RAD3* and *SSL2* genes, designated *ERCC2* and *ERCC3*, respectively, have been clearly implicated in the human hereditary diseases xeroderma pigmentosum (XP) and Cockayne syndrome (CS) (7–9).

We have recently shown (10) that the product of the *RAD3* gene is the 85-kDa subunit of RNA polymerase II transcription initiation factor b, which is the yeast homolog of the human basal transcription factor TFIIF (also called BTF2) (11). These studies also demonstrated that, while the *SSL2* gene product is not a component of purified factor b, *in vitro*-translated *SSL2* protein binds to this factor (10). It has been independently shown that TFIIF (BTF2) purified from human cells includes the human homolog of *SSL2* protein, *ERCC3* (12). The 50-kDa subunit of factor b has been identified as the product of the *SSL1* gene (10). This gene too has been shown to be essential for the viability of yeast cells, and viable alleles that confer increased sensitivity to killing by ultraviolet (UV) radiation have been identified (13), sug-

gesting that it might also be involved in NER. Factor b also contains the 75-kDa *TFB1* gene product (11) and additional subunits with apparent molecular masses of 55 and 38 kDa (ref. 10; J.Q.S., unpublished data).

In the past several years we (E.C.F. and colleagues) have undertaken a systematic examination of interactions between yeast NER proteins. We have previously reported that the *RAD1* and *RAD10* gene products form a stable and specific complex, both *in vitro* and in living cells (14, 15). Here we describe a series of protein–protein interactions involving the *RAD3*, *SSL2*, *SSL1*, and *TFB1* gene products, with implications for the architecture and function of the factor b–*SSL2* transcription–repair complex.

MATERIALS AND METHODS

Yeast Strains. Strains GGY::171 and CTY10-5d were gifts from S. Fields (State University of New York). Strain Y187 was a gift from S. Elledge (Baylor College of Medicine). Strains GGY::171 and Y187 contain chromosomal integrants of the *Escherichia coli lacZ* gene under the control of the yeast *GAL1* promoter and activated by the upstream activation sequence UAS_G, the target of the yeast *GAL4* protein DNA-binding domain. CTY10-5d contains a chromosomal integrant carrying four binding sites for *E. coli* LexA dimers upstream of the transcription start site of a *GAL1-lacZ* gene from which the UAS_G was deleted.

Transcription Plasmid Constructions. A region from the start codon through the internal *Bam*HI site was amplified from a *RAD3*-containing plasmid in a polymerase chain reaction by using the primers 5'-CGGGATCCACCAT-GAAGTTTATATAGATGAT and 5'-CGGGCATGCGAAATCATACGACGAACAAT, was digested with *Bam*HI and *Hind*III, and was reconstructed with the *Hind*III–*Sal*I fragment of *RAD3* from pGALRAD3 (16) into *Bam*HI/*Sal*I-cut pGEM-4Z (Promega Biotech) to generate pGEM-4Z-SP6RAD3. *SSL2* was amplified from yeast genomic DNA by using the primers 5'-GGGGGATCCATGACGGACGT-TGAAGGCTA and 5'-GGGGGATCCTGAAACCAAGCC-TATTCACCTT and was inserted into *Bam*HI-cut pGEM-4Z to generate pGEM-4Z-SP6SSL2 and pGEM-4Z-T7SSL2. *SSL1* was amplified by using the primers 5'-GCGAATTCG-GATCCACCATGGCTCCTGTAGTTATTCA and 5'-GCGAATTCGTCGACAGATTTTCGTTAAGTTATTA and was inserted into *Eco*RI-cut pGEM-4Z to generate pGEM-4Z-T7SSL1. *TFB1* was amplified by using the primers 5'-CGCGGATCCACCATGTCACATTCCGGAGCTGCCA and 5'-CGCGGATCCTTAAGTTTTATGTAATATG-

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Abbreviations: NER, nucleotide excision repair; XP, xeroderma pigmentosum; CS, Cockayne syndrome.

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TAACA and was inserted into *Bam*HI-cut pGEM-4Z to generate pGEM-4Z-SP6TFB1 and pGEM-4Z-T7TFB1.

Two-Hybrid Plasmid Constructions. Two-hybrid constructions encoded fusion proteins consisting of yeast GAL4 or *E. coli* LexA amino acid sequences, followed by a short spacer of amino acids derived mainly from polylinker sequences; these DNA constructions were followed by the entire open reading frame of the indicated *RAD*, *SSL*, or *TFB1* gene, subcloned from the transcription plasmids described above. pMA424, pGAD.1F (pGAD-SSL2), and pGAD10 (pGAD-TFB1) have been described (15). Plasmids pGBT9 and pBTM116 (from P. Bartel and S. Fields, State University of New York) are multicopy vectors that contain the sequence encoding the GAL4 DNA-binding domain or LexA, respectively, expressed from the *ADH* promoter. pGADHB (pGAD-RAD3, pGAD-SSL1) was derived from pGADGH (from G. Hannon, Cold Spring Harbor Laboratories). The polylinker of pGADGH between the *Spe* I and *Sal* I sites was replaced by oligonucleotide 5'-CTAGTCGAATTCGAGC-TCGGATCCCCGGG annealed to 5'-TCGACCCGGGATCCGAGCTCGAATTCGA. The resulting multicopy vector (pGADHB) contains the sequence encoding the GAL4 activation domain expressed from the *ADH* promoter. pBTMR4ins (used as a negative control) encodes a fusion protein of *E. coli* LexA bound to the N-terminal third of yeast RAD4 protein.

Assays for Activation of *lacZ* Transcription. Filter and quantitative liquid assays for β -galactosidase activity were carried out as described (15).

Antisera, Protein Purification, and Buffers. The production and affinity purification of the antisera used in this study have been described (11, 16, 17). The purification of factor b will be described elsewhere (J.Q.S., unpublished data). RAD3 protein was purified as described (18). Buffers B and C have been described (14).

Transcription, Translation, and Immunoprecipitation. *In vitro* transcription and translation, the partial purification of translation products, and immunoprecipitations were essentially as described (14). RAD3 affinity beads (see below and Fig. 2) bound to *in vitro*-translated proteins (see Fig. 2) were washed 5 min in buffer B (14), 5 min in buffer C (14), 5 min in buffer B containing 300 mM KCl, and then 5 min in buffer B. Our calculation of the limit of sensitivity of the immunoprecipitation assay relied on the following premise: to score an interaction as positive, we required that the radioactive signal be at least 3-fold above the background level observed in the presence of antiserum but in the absence of antigen (the background was rarely less than 0.1% of the total radioactivity in the reaction).

RESULTS

Interactions Between RAD3, SSL1, and TFB1 Proteins *in Vivo*. We initially searched for pairwise interactions among RAD3, SSL2, SSL1, and TFB1 proteins using the "two-hybrid" genetic assay (19), which can detect interactions between two proteins in living cells. When one protein, expressed as a GAL4 DNA-binding domain fusion protein, interacts with a second, expressed as a GAL4 activation domain fusion protein, the proteins can reconstitute GAL4 function, which is assayed by monitoring transcription from a reporter *lacZ* gene. The DNA-binding domain is encoded by plasmids pGBT9 and pMA424 and the activation domain is encoded by pGAD plasmids. The GAL4 DNA-binding domain fusion can be expressed as a LexA protein fusion by cloning into the vector pBTM116. In this case *lacZ* is activated from upstream LexA-binding boxes.

When either plasmid pBTM-RAD3 or pGBT-RAD3 was cotransformed with plasmid pGAD-SSL1 (Fig. 1 *Upper Right*), colonies turned deep blue within 3–5 hr in the filter

Fig. 1. Interactions between RAD3 and SSL1 proteins and SSL1 and TFB1 proteins in the two-hybrid assay. Transformants containing pGBT-RAD3 + pGAD-TFB1 (*Upper Left*), pGBT-RAD3 + pGAD-SSL1 (*Upper Right*), pMA-SSL1 + pGAD-TFB1 (*Lower Left*) and pMA-SSL1 + pGAD-SSL1 (*Lower Right*) were transferred to nitrocellulose filters, which were immersed in liquid nitrogen and incubated in the presence of 5-bromo-4-chloro-3-indolyl β -D-galactoside indicator. A positive interaction is indicated by colonies turning blue.

assay, indicating an interaction between the RAD3 and SSL1 proteins. No interaction between the RAD3 and SSL2 proteins or the RAD3 and TFB1 proteins (Fig. 1 *Upper Left*) or self-interaction of RAD3 protein was observed. The RAD3–SSL1 interaction was detected regardless of whether the RAD3–GAL4 DNA-binding domain or the RAD3–LexA DNA-binding domain fusions were tested. When pMA-SSL1 was cotransformed with pGAD-RAD3, the interaction was considerably weaker. The observation that the strength of the interaction between RAD3 and SSL1 protein varied depending on which protein was expressed as the DNA-binding domain fusion is similar to results obtained for the RAD1–RAD10 interaction (15).

When cells were transformed with pMA-SSL2 plus pGAD-SSL1 (Fig. 1 *Lower Right*) or with pMA-SSL1 and pGAD-SSL2, no detectable interaction between SSL2 and SSL1 proteins was observed *in vivo*. However, when pMA-SSL1 was cotransformed with either pGAD-TFB1 (Fig. 1 *Lower Left*) or with pGAD-SSL1, both combinations led to transcriptional activation of *lacZ*. Thus, SSL1 can interact with itself *in vivo* and also can complex with TFB1 protein. We note that in independent studies (10), SSL1 has been identified in a library screen for proteins that interact with GAL4-TFB1 fusion proteins. No interactions between RAD3, SSL1, SSL2, or TFB1 and either RAD1 or RAD10 were detected in this assay (data not shown). Table 1 shows relative β -galactosidase activities for the RAD3–SSL1, SSL1–SSL1 and SSL1–TFB1 interactions determined in a quantitative liquid assay (15).

RAD3 Binds Directly to SSL1 and SSL2 Proteins *in Vitro*. To determine whether SSL2, SSL1, or TFB1 proteins could interact with purified RAD3 protein, we generated RAD3 affinity beads by binding purified RAD3 protein to anti-RAD3 antibody-conjugated protein A-agarose beads. For controls, RAD3 antibodies were bound to protein A-agarose beads in the absence of RAD3 protein, or RAD3 protein was "bound" to the beads in the absence of RAD3 antibodies. The beads were incubated with SSL1, TFB1, or SSL2 proteins that had been translated and radiolabeled *in vitro*. As shown in Fig. 2, both *in vitro*-translated SSL1 protein (compare lane 1 to lanes 2 and 3) and SSL2 protein (compare lane 7 to lanes 8 and 9) bound to the RAD3 affinity beads. The weak binding of TFB1

Table 1. Quantitative measurement of protein–protein interactions assayed by β -galactosidase activity

Domain-encoding plasmids		Incubation time, min at 37°C	β -Galactosidase activity	
DNA-binding domain	Activation domain		Miller units	Fold increase
pGBT9	pGADF	20	3.7	1.0
pGBT-RAD3	pGADF	20	4.2	1.1
pGBT9	pGAD-SSL1	20	5.8	1.6
pGBT-RAD3	pGAD-SSL1	20	52.0	14.1
pBTMR4ins	pGADF	20	3.4	1.0
pBMT-RAD3	pGADF	20	3.3	1.0
pBTMR4ins	pGAD-SSL1	20	5.0	1.5
pBTM-RAD3	pGAD-SSL1	20	50.7	14.9
pMA424	pGADF	20	4.3	1.0
pMA424	pGAD-SSL1	20	7.0	1.6
pMA-SSL1	pGADF	20	6.7	1.6
pMA-SSL1	pGAD-SSL1	20	47.9	11.1
pMA424	pGAD-TFB1	20	5.4	1.3
pMA-SSL1	pGAD-TFB1	20	35.0	8.1
pMA-TFB1	pGADF	20	20.3	4.7
pMA-TFB1	pGAD-SSL1	20	1,242.7	289.0
pMA424	pGADF	200	0.6	1.0
pMA-SSL1	pGADF	200	0.8	1.3
pMA424	pGAD-RAD3	200	0.7	1.2
pMA-SSL1	pGAD-RAD3	200	1.8	3.0

protein (lane 4) is <3-fold above background and is not interpreted as a significant interaction (see *Materials and Methods*). SSL1-(1–251) truncation polypeptide (comprising the N-terminal 251 amino acids) did not interact with RAD3 protein in this assay (data not shown), indicating that the C-terminal half of SSL1 protein is required for its proper folding and/or its binding to RAD3. Detection of RAD3–SSL1 and RAD3–SSL2 binding in this assay required a relatively high concentration of RAD3 protein (≈ 50 nM). When the concentration of RAD3 was reduced by 1 order of magnitude, these interactions were no longer detectable above background (data not shown). We have calculated that the equilibrium dissociation constants for these pairwise interactions are >500 nM under the conditions used.

Factor b Binds with High Affinity to SSL2 Protein. We have recently shown that *in vitro* translated SSL2 protein immunoprecipitates with highly purified factor b in the presence of antisera to TFB1 protein or to RAD3 protein (10). To further explore the specificity of this interaction, SSL2, RAD1, and RAD10 proteins were translated *in vitro* and mixed in various combinations with purified factor b in immunoprecipitation

reactions (Fig. 3). When RAD1, RAD10, and SSL2 proteins were added to the same binding reaction, SSL2 protein coprecipitated with factor b but RAD1 and RAD10 proteins did not (Fig. 3, lanes 2 and 3). As shown previously (14), RAD1 protein coprecipitated with RAD10 protein in the presence of RAD10 antisera (Fig. 3, lanes 4 and 5). However, SSL2 protein did not coprecipitate with the RAD1/RAD10 complex either in the absence (Fig. 3, lane 5) or presence (Fig. 3, lane 6) of factor b. By quantitative assay (14), the equilibrium dissociation constant for the SSL2–factor b interaction was measured at 125 nM, and the half life was determined to be >15 min (data not shown). The addition of 5 mM ATP, ADP, or the nonhydrolyzable analog adenosine 5'-[γ -thio]triphosphate to the binding reactions did not significantly affect the SSL2–factor b binding equilibrium (data not shown).

FIG. 2. SSL1 and SSL2 proteins interact with RAD3 affinity beads *in vitro*. Purified RAD3 protein (250 ng) and anti-RAD3 antibody (30 μ g) (lanes 1, 4, and 7) or just antibody (lanes 2, 5, and 8) or RAD3 protein (lanes 3, 6, and 9) was incubated with protein A-agarose beads (15 μ l of slurry). After extensive washing, the beads were used in an immunoprecipitation reaction with 500 fmol of 35 S-labeled SSL1 (lanes 1–3), TFB1 (lanes 4–6), or SSL2 protein (lanes 7–9) and 20 μ g of unlabeled reticulocyte lysate proteins. Bound proteins were eluted and visualized by SDS/PAGE (10% acrylamide) followed by autoradiography. The positions of full-length proteins are shown on the left and of molecular mass standards (in kDa) on the right.

FIG. 3. Factor b binds to SSL2 but not the RAD1–RAD10 complex. *In vitro*-translated SSL2, RAD1, and RAD10 proteins were loaded (5 fmol of labeled protein per lane) directly onto an SDS/10% PAGE gel (lanes 1, 7, and 8) or were immunoprecipitated with anti-TFB1 (lanes 2 and 3) or anti-RAD10 sera (lanes 4–6) in the presence (lanes 3 and 6) or absence (lanes 2, 4, and 5) of 1 pmol of factor b prior to gel analysis. Immunoprecipitation reaction mixtures contained 1 pmol of 35 S-labeled RAD10 protein (lanes 2, 3, 5, and 6) and/or 1 pmol of SSL2 protein plus 250 fmol of RAD1 protein (lanes 2–6) and 40 μ g of unlabeled reticulocyte lysate proteins. The positions of molecular mass standards (in kDa) are shown on the right.

The N-Terminal Half of SSL2 Protein Is Sufficient for Binding Factor b. The mutation in one of the *ERCC3* alleles from XP/CS patient XP11BE results in a protein that differs from the wild type in the C-terminal 42 amino acids (9). Two different *ssl2* alleles have been constructed to mimic this mutation in yeast cells. The alleles *ssl2*₁₋₇₄₉ [also called *ssl2*-XP (4)] and *ssl2*₁₋₇₉₇ [also designated *rad25*_{799am} (5)] are abnormally sensitive to UV radiation but support normal cell growth. The C-terminal truncation polypeptide SSL2-(1-749) interacted with factor b with the same efficiency as did the full-length SSL2 protein (Fig. 4, lanes 4–7). A ladder of lower molecular weight fragments also coprecipitated with factor b. These presumably derive from premature translational termination of SSL2 mRNA, since their positions were unchanged in SSL2-(1-749) (compare lanes 5 and 7 in Fig. 4). This result suggests that the factor b-binding domain of SSL2 protein is contained in the N-terminal half of the polypeptide. Indeed, the polypeptide SSL2-(1-379), consisting of only the N-terminal 45% of SSL2 protein, was also able to bind to factor b (Fig. 4, lanes 8 and 9).

The DNA-Repair Function of SSL2 Is Not Dependent on the Essential Function. We confirmed expression of the pGAD-SSL2 hybrid construct by Western blotting (immunoblotting) with antisera to the C-terminal portion of GAL4 protein (data not shown). Furthermore, we demonstrated that both the pMA-SSL2 and pGAD-SSL2 fusion constructs complemented the UV radiation sensitivity of an *ssl2* mutant strain. However, in plasmid shuffling experiments, we were unable to rescue the viability of *ssl2* deletion mutants with these plasmids, indicating that they are unable to complement the essential function of *SSL2* (data not shown). In contrast, the GAL4-RAD3 fusions, and the LexA-RAD3 fusion, complemented both the UV sensitivity and the lethality of relevant *rad3* alleles (data not shown). Hence, fusions of heterologous domains to the N terminus of SSL2 represent a novel class of *ssl2* alleles in which the essential function is compromised but the DNA repair function is preserved, indicating that the DNA repair function is not dependent on the essential function.

DISCUSSION

We have demonstrated a series of pairwise interactions involving the RAD3, SSL2, SSL1, and TFB1 polypeptides. SSL1 protein interacted with itself, with RAD3 protein, and with TFB1 protein in the nucleus of living cells, as revealed by the two-hybrid assay. Furthermore, RAD3 affinity beads bound directly to both SSL1 protein and SSL2 protein *in vitro*. Neither factor b, its identified components, nor SSL2

interacted with the RAD1–RAD10 complex in either the two-hybrid or immunoprecipitation assay.

The *SSL1* and *SSL2* genes were both isolated in a selection designed to identify genes involved in translation initiation (4, 13). Genetic interactions between alleles of these genes led to the expectation that the SSL1 and SSL2 proteins might bind to each other (4). Our data provide no direct support for this; instead we find that RAD3 protein binds to both of these proteins. Hence, RAD3 may mediate the genetic interactions between *SSL1* and *SSL2*. The observations that RAD3 protein coprecipitates with TFB1 protein from cell-free extracts and vice versa (data not shown) and that both SSL1 and RAD3 proteins copurify with TFB1 protein and with a basal transcription activity defined as factor b (10) suggest that SSL1 protein can bind simultaneously to both TFB1 and RAD3 proteins.

The interaction of SSL2 protein with factor b is relatively stable with a calculated $K_d \approx 125$ nM and a half life of >15 min. SSL2 protein bound to RAD3 affinity beads with a lower affinity ($K_d > 500$ nM). This suggests that SSL2 may contact another subunit(s) of factor b in addition to RAD3. Alternatively, the structure of RAD3 protein when bound in the factor b complex may facilitate more efficient binding to SSL2 protein. It should be noted, however, that these interpretations may be complicated by the effects of the polyclonal antisera on the binding equilibria. The N-terminal half of SSL2 protein is sufficient for the interaction with factor b. Interestingly, this region of the protein is devoid of the helicase signature motifs, which are all located in the C-terminal half of SSL2 protein. The DNA repair defect of C-terminal truncation alleles of *SSL2* is apparently not due to a defective interaction with factor b. The protein interactions mapped in this study begin to suggest a quaternary structure of factor b and the factor b–SSL2 complex (Fig. 5).

RAD3 protein is a processive 5' → 3' DNA-DNA and DNA-RNA helicase (2, 3, 20). The predicted helicase activity of SSL2 protein has yet to be verified biochemically, though such verification has been provided for its highly conserved human homolog, ERCC3 (12). With this caveat in mind, the interaction between RAD3 and SSL2 proteins is, to our knowledge, the first demonstration of a direct heteromeric interaction between two eukaryotic helicases. Biochemical evidence suggests that RAD3 protein may patrol the DNA strand for the presence of damaged bases (18, 21). The possibility that such an activity might be closely coupled to the biochemical activity of SSL2 protein must now be entertained. The binding of the RAD3 helicase to the zinc-finger-containing SSL1 protein has further functional implications. If SSL1 protein indeed interacts with DNA, it could modulate the damage recognition component or the processivity of RAD3, while the helicase activity of RAD3 protein could impart a vectorial component to the SSL1–DNA interaction.

GAL4–SSL2 fusions can complement the UV sensitivity of *ssl2*-XP mutants but not the essential function of *ssl2* deletion mutants. Since the essential function of RAD3 and SSL2 proteins may be in transcription, the inviability of GAL4–SSL2 fusion proteins may reflect a blocked binding interaction with RAD3 protein by the presence of a GAL4 domain at the amino terminus of SSL2. Regardless of the precise mechanisms, this provides further evidence that the essential and repair functions of *SSL2* are genetically separable and

FIG. 4. SSL2 deletion proteins interact with factor b. *In vitro* translated SSL2 full-length and deletion proteins were loaded (5 fmol of labeled protein per lane) on an SDS/10% PAGE gel (lanes 1–3) or were immunoprecipitated with TFB1 antisera (lanes 4–9) in the presence (lanes 5, 7, and 9) or absence (lanes 4, 6, and 8) of 1 pmol of factor b prior to gel analysis. Immunoprecipitation reactions contained 1 pmol of ³⁵S-labeled SSL2 full-length or deletion proteins and 40 μ g of unlabeled reticulocyte lysate proteins. Molecular mass standards (in kDa) are shown on the right.

FIG. 5. Possible architecture of the factor b–SSL2 complex. See text for details.

suggests that SSL2 protein has a direct function in NER rather than an indirect role subserved by the transcription of (other) repair genes. Hence, we believe it likely that the *RAD3* and *SSL2* genes participate directly in NER. This conclusion was recently supported by independent biochemical studies (24). However, we do not exclude the possibility that mutant alleles of the human homologs of these genes may contribute to the pathology of XP and CS by altering gene expression, as previously suggested (4, 12, 22, 23).

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