MOLECULAR AND CELLULAR BIOLOGY, Dec. 1997, p. 6803–6814 Vol. 17, No. 12
0270-7306/97/$04.00+0


# Recruitment of the Putative Transcription-Repair Coupling Factor CSB/ERCC6 to RNA Polymerase II Elongation Complexes

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Received 9 July 1997/Returned for modification 12 August 1997/Accepted 10 September 1997

**Cockayne's syndrome (CS) is a disease characterized by developmental and growth defects, sunlight sensitivity, and a defect in transcription-coupled nucleotide excision repair. The two principle proteins involved in CS, CSA and CSB/ERCC6, have been hypothesized to bind RNA polymerase II (Pol II) and link transcription to DNA repair. We have tested CSA and CSB in assays designed to determine their role in transcription-coupled repair. Using a unique oligo(dC)-tailed DNA template, we provide biochemical evidence that CSB/ERCC6 interacts with Pol II molecules engaged in ternary complexes containing DNA and nascent RNA. CSB is a DNA-activated ATPase, and hydrolysis of the ATP β-γ phosphoanhydride bond is required for the formation of a stable Pol II-CSB-DNA-RNA complex. Unlike CSB, CSA does not directly bind Pol II.**

A cell's genetic information can be replicated, recombined with other DNA molecules, and expressed in the form of RNA. It must furthermore be defended against continuous attack from internal and external DNA-damaging agents. These nuclear events must also take place within the framework of the cell cycle and its control. A growing body of information indicates that all of these processes, mediated variously by DNA and RNA polymerases, recombinases, the multiple DNA repair systems, and the cell cycle machinery, are intimately linked. Transcription and replication, for example, are known to be closely coregulated (1, 4, 10, 34, 37, 38). Other recent reports indicate a close mechanistic relationship between replication and recombination (23) and a negative relationship between cell division and transcription (47).

Hanawalt and coworkers originally identified an interaction between transcription and DNA repair when they found that in cultured hamster and human cells, UV radiation-induced pyrimidine dimers were repaired in the actively transcribed dihydrofolate reductase gene at a higher rate than the photodimers in surrounding DNA (5, 31). This phenomenon, known as transcription-coupled repair (TCR), was largely restricted to the transcribed strand of the active gene in human cells, indirectly implicating RNA polymerases in the process (33). Experiments later extended TCR to *Escherichia coli* (32), and an activity dubbed transcription-repair coupling factor (TRCF) that facilitated TCR in vitro was isolated (48). TRCF, the product of the previously described *mfd* gene (50), was shown to displace stalled bacterial RNA polymerase and interact with the UvrA nucleotide excision repair (NER) protein, providing a mechanism of TCR in prokaryotic cells. TCR has also been described for the yeast *RPB2* and *GAL7* genes, where it has been shown to require an actively transcribing polymerase (27, 51). Nothing is known, however, of the molecular mechanism of facilitated repair in eukaryotes.

More recent evidence for the link between transcription and DNA repair came from studies of the eukaryotic RNA polymerase II (Pol II) general transcription factor IIH (TFIIH). The well-documented energetic requirements for Pol II transcription initiation (6, 43) appear to directly involve TFIIH. TFIIH mediates ATP-dependent DNA melting at the transcription start site to form an open complex (17, 60). Some negatively supercoiled promoters do not require TFIIH in vitro (40) and also circumvent the energetic requirement for ATP (54). Templates bearing heteroduplex start sites also eliminate the requirements both for TFIIH and for hydrolysis of the ATP β-γ phosphoanhydride bond (16, 39, 52).

Cloning of the genes encoding the two TFIIH helicases revealed that they were identical to the XPB/ERCC3 and XPD/ERCC2 NER proteins (44, 45). The other core subunits of TFIIH were all subsequently found to be important NER factors (9, 18, 29, 61). Mammalian and yeast TFIIHs were shown to be important repair factors, because they could complement NER-defective cell extracts (9, 62). The yeast TFIIH was shown to complement a *rad3* extract, whereas the isolated Rad3 protein could not (62), indicating that Rad3 operated only in the context of the intact complex.

In humans, mutations in various TFIIH subunits can cause xeroderma pigmentosum (XP), trichothiodystrophy, or XP with manifestations of Cockayne's syndrome (CS). XP is a defect in NER. Trichothiodystrophy is a complex disease also referred to as brittle hair syndrome. CS is another complex disease characterized by sunlight sensitivity, mental retardation, and developmental defects (reviewed in reference 28). Which disease phenotype is elicited depends on the specific mutation in the TFIIH subunit. Two fundamental observations were that cells taken from CS patients are sensitive to various DNA-damaging agents, such as UV radiation and the UV-mimetic agent NA-AAF (46, 58), and that they do not recover the ability to synthesize RNA after exposure to UV radiation (30, 57). CS cells also lack the kinetic preference for repair of the transcribed strands of transcriptionally active genes for many types of DNA damage (26, 57, 59) and are thus defective in TCR.

Interestingly, the majority of CS patients do not have disease associated with XP but have mutations in one of two genes, known as *CSA* and *CSB*/*ERCC6* (reviewed in reference 12). These genes, which have both been cloned (14, 55), encode

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proteins (also termed CSA and CSB) that have been hypothesized to be functional eukaryotic homologs of the prokaryotic TRCF (12, 15, 42). Another study has questioned this idea and instead proposed that CSA and CSB function in the interconversion of TFIIH between transcription- and NER-active forms (58). *CSB* encodes a large protein of 168 kDa that, like the *mfd* gene product, contains a series of helicase motifs, although the helicase motifs in CSB show more homology to proteins such as SWI2 (55) and MOT1 (unpublished observations) than they do to the prokaryotic TRCF. The function of the CSB helicase motifs is unknown. The 44-kDa CSA protein contains a series of WD, or β-transducin, repeats, which are believed to mediate protein-protein interactions (36).

Previous studies have employed CSA and CSB synthesized in vitro to show that they interact with one another, that CSA interacts with the TFIIH p44 subunit (14), and that CSB interacts with the TFIIH-associated factor XPG (19). An interaction between XPA and in vitro-translated CSB has also been described (49). CSB and its yeast homolog Rad26 (56) have recently been overexpressed and purified from baculovirus-infected insect cells (49) or directly from yeast (13). Although no helicase activity was detected, both proteins contain a DNA-stimulated ATPase activity, as does the *E. coli* TRCF. The functional role of CSB's ATPase activity is unknown.

We sought to evaluate models for TCR which postulate that CSA and/or CSB acts as a coupling factor, interacting simultaneously with transcribing Pol II molecules and NER factors such as TFIIH. CSB is an ATPase that is marginally stimulated by the CSA protein. Using purified recombinant human CSB and an oligo(dC)-tailed template containing a transcribing Pol II molecule, we show that CSB interacts with a ternary complex containing DNA, RNA, and stalled Pol II in a manner that requires ATP β-γ bond hydrolysis.

## MATERIALS AND METHODS

**Subcloning of CSB and expression in Sf9 cells.** The CSB cDNA was cloned into the expression vector pBLUEBACHisA (Invitrogen) in two steps. In the first step, a fragment of the plasmid pBISE6 (a gift of J. H. J. Hoeijmakers) was amplified by PCR with primers that contained *Bam*HI sites at their 5′ ends (primer CSB-Bam5′, 5′CCGGATCCATGCCAAATGAGGGAA3′, primer CSB-Bam3′, 5′CCGGATCCGCATGCTGCCAAGACT3′). This fragment was then digested with *Bam*HI and ligated into the *Bam*HI site of pBLUEBACHisA, generating pBBH-ΔCSB. pBISE6 was then digested with *Sac*I and *Sal*I, generating an approximately 4-kb fragment that contained the majority of the CSB-coding sequence. The recessed 3′ end of the *Sal*I site was filled in with Klenow polymerase (Gibco BRL), and this fragment was ligated into the unique *Sac*I site in the CSB insert in pBBH-ΔCSB, generating pBBH-CSB. The orientation was verified by PCR, and the final coding sequence was confirmed by direct DNA sequencing. Tissue culture, transformation, and virus purification were performed by using the pBLUEBACHis expression system (Invitrogen) and attendant products exactly as stipulated by the supplier. Transfections were performed by using Insectin (Invitrogen), as described by the supplier.

**Expression and purification of recombinant CSA and CSB.** CSB was expressed and purified from Sf9 spinner cultures infected for 50 h with recombinant virus at a multiplicity of infection of 5:1. Cells were harvested by centrifugation at 4°C for 5 min at 3,500 rpm in a GSA rotor. Pellets from 250 ml of harvested cells (approximately $2.5 \times 10^8$ cells) were resuspended in 25 ml of buffer 1 (20 mM $NaPO_4$ [pH 7.3], 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], and 1 μg of leupeptin per ml), and the cells were freeze-thawed twice in dry ice-ethanol and a 39°C water bath. This suspension was briefly disrupted for 5 to 10 s on ice by using a Heat Systems ultrasonicator with a microprobe at power setting 4. After centrifugation in an SS-34 rotor at 10,000 rpm for 20 min at 4°C, the lysates were passed through a 7-ml DEAE-Sepharose Fast Flow Column (Pharmacia) equilibrated in buffer 1. The flowthrough was loaded onto a 4.5-ml double-stranded-DNA–cellulose column (Gibco BRL) equilibrated in buffer 1 and washed with buffer 2, in which EDTA was omitted and 10 μM 2-mercaptoethanol (βME) was substituted for DTT. The column was developed with native binding buffer (NBB) (20 mM $NaPO_4$ [pH 7.3], 500 mM NaCl, 10 μM βME, 0.5 mM PMSF, 1 μg of leupeptin per ml). CSB was located in the peak fraction, which was loaded directly onto a 0.75-ml nickel-nitrilotriacetic acid-agarose (Qiagen) column equilibrated in NBB. The column was washed with NBB and native wash buffer (NWB) (20 mM $NaPO_4$ [pH 6.4], 500 mM NaCl, 10 μM βME, 0.5 mM PMSF, 1 μg leupeptin per ml) and developed subsequently with NWB plus 20, 50, 100, and 200 mM imidazole. CSB eluted at 100 and 200 mM imidazole. Fractions containing CSB, as visualized by Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels, were pooled, DTT and bovine serum albumin (BSA) were added to 1 mM and 0.2 mg/ml, respectively, and the sample was concentrated and desalted with Centricon-100 concentrators, using a protocol supplied by Amicon. The identity and purity of the fractions were confirmed by SDS-polyacrylamide gel electrophoresis, followed by Coomassie blue staining or Western blotting with anti-enterokinase (anti-EK) epitope antibodies (Invitrogen).

The CSA expression plasmid was a kind gift of E. C. Friedberg. CSA was expressed and purified from *E. coli* lysates that had been induced for 4 h with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG). The lysates were loaded onto a 1.5-ml Ni-nitrilotriacetic acid-agarose column equilibrated in NBB and washed extensively. After equilibration in NWB, the column was developed with NWB containing 20, 50, and 100 mM imidazole. The most pure and concentrated form of CSA eluted at 50 mM imidazole. Fractions collected from the 50 mM elution were pooled and dialyzed against transcription buffer (12 mM HEPES [pH 7.9], 0.12 mM EDTA, 12% glycerol, 60 mM KCl, 1 mM DTT, 0.5 mM PMSF). At this stage of the purification, CSA was found to have a contaminating single-stranded-DNA-dependent ATPase activity. Therefore, 2.0 ml of the dialyzed fraction was loaded onto a 0.8-ml single-stranded-DNA–cellulose column (Pharmacia) equilibrated in buffer D (20 mM HEPES [pH 7.9], 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF) containing 100 mM KCl. The column flowthrough contained no detectable ATPase activity but still contained the CSA protein as judged by Coomassie blue staining.

**Purification of Pol II.** Pol II was immunopurified from HeLa nuclear pellets by using a combination of two previously published protocols (41, 53). Briefly, three 32-liter cultures of HeLa spinner cells were prepared for nuclear extracts by the procedure of Dignam et al. (8). The HeLa nuclear pellets derived from this procedure were resuspended in 170 ml of ice-cold buffer (50 mM Tris-Cl [pH 7.9], 25% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 0.5 mM PMSF), placed on ice, and homogenized with a Dounce homogenizer to an even suspension. Next, 3.8 M $(NH_4)_2SO_4$ was slowly added to a final concentration of 0.3 M, during which the solution became viscous. The approximately 230-ml suspension was then disrupted with an ultrasonicator at power setting 9 for 4 min in 20-s bursts and centrifuged at 40,000 rpm in a Ti-45 rotor for 60 min at 4°C. The supernatant was collected, and 0.42 g of solid $(NH_4)_2SO_4$ per ml was added over a 30-min period with stirring on ice. After 30 min of additional stirring, the suspension was centrifuged at 40,000 rpm for 55 min at 4°C. The pellet was resuspended in buffer D (no salt) until the final conductivity matched that of a 145 mM $(NH_4)_2SO_4$ salt solution. This mixture was centrifuged at 40,000 rpm for 1 h at 4°C.

The supernatant was loaded onto a 60-ml DEAE-cellulose column (Whatman) equilibrated in buffer D plus 0.145 mM $(NH_4)_2SO_4$, washed, and developed with buffer D containing 500 mM $(NH_4)_2SO_4$. The peak fraction, which contained Pol II, was dialyzed into 8WG16 binding buffer [20 mM Tris-Cl (pH 7.4), 1 mM EDTA, 200 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.5 mM PMSF), and the insoluble material was removed by centrifugation at 8,000 rpm in a GSA rotor at 4°C. One-half of this preparation was bound to an affinity resin generated from 2 ml of protein A-Sepharose (Pharmacia) that had been covalently cross-linked to antibodies from an ascites fluid containing the 8WG16 monoclonal antibody (53) for 3.5 h at 4°C. The bound beads were collected by low-speed centrifugation in an IEC clinical centrifuge at 4°C, washed sequentially with 20, 10, and 10 ml of ice-cold 8WG16 binding buffer, and then incubated with 5 ml of 8WG16 wash buffer [20 mM Tris-Cl (pH 7.4), 1 mM EDTA, 500 mM $(NH_4)_2SO_4$, 30% ethylene glycol, 1 mM DTT, 0.5 mM PMSF) for 20 min at room temperature with gentle agitation. The supernatant was collected, and this process was repeated five times. The supernatants from the first and second elutions were dialyzed against transcription buffer. The final three elutions were pooled, dialyzed against buffer D plus 0.1 M $(NH_4)_2SO_4$, loaded onto a 2-ml DEAE-cellulose column, developed as before, and dialyzed against transcription buffer. These preparations appeared to be homogeneous and contained each of the identified subunits of Pol II as judged by silver staining (data not shown). The first and second elutions contained mostly Pol IIA and an approximately 50:50 mixture of Pol IIA and Pol IIO, respectively. The concentrated preparation from elutions 3 to 5 contained mostly Pol IIO.

**Preparation of C-tailed templates or probes.** High-pressure liquid chromatography-purified oligonucleotides were purchased from Operon. The oligonucleotide GAL4staCOD is a 72-mer (5′GACCCGGAGTACTGTCCTCCGCTCTTTTTTCCCTTTTTTCTATACCACCCTTTTACTCTCCTTTACCCAAT3′). The oligonucleotide GAL4sta TEM is a 92-mer (5′ATTGGGTAAAGGAGAGTATTTGGGTGGTATAGAAAAAAGGGAAAAAAAGAGCGGAGGACAGTACTCCGGGTCCCCCCCCCCCCCCCCCCCC3′). Underlined residues indicate the position of the heteroduplex. The template was created by mixing 40 pmol of GAL4staCOD and 40 pmol of unlabeled or 5′-$^{32}$P-labeled GAL4staTEM together in a 200 μl annealing mixture containing 20 mM Tris-Cl (pH 7.4), 1 mM EDTA, and 0.2 M NaCl. The mixture was heated to 90°C for 4 min, cooled slowly to 55°C, and held at that temperature for 2 h, after which it was allowed to cool slowly to room temperature. For the damaged template, 5 μl of 57 mM $KMnO_4$ was added to one-half (100 μl, 20 pmol of annealed probe) of the annealed

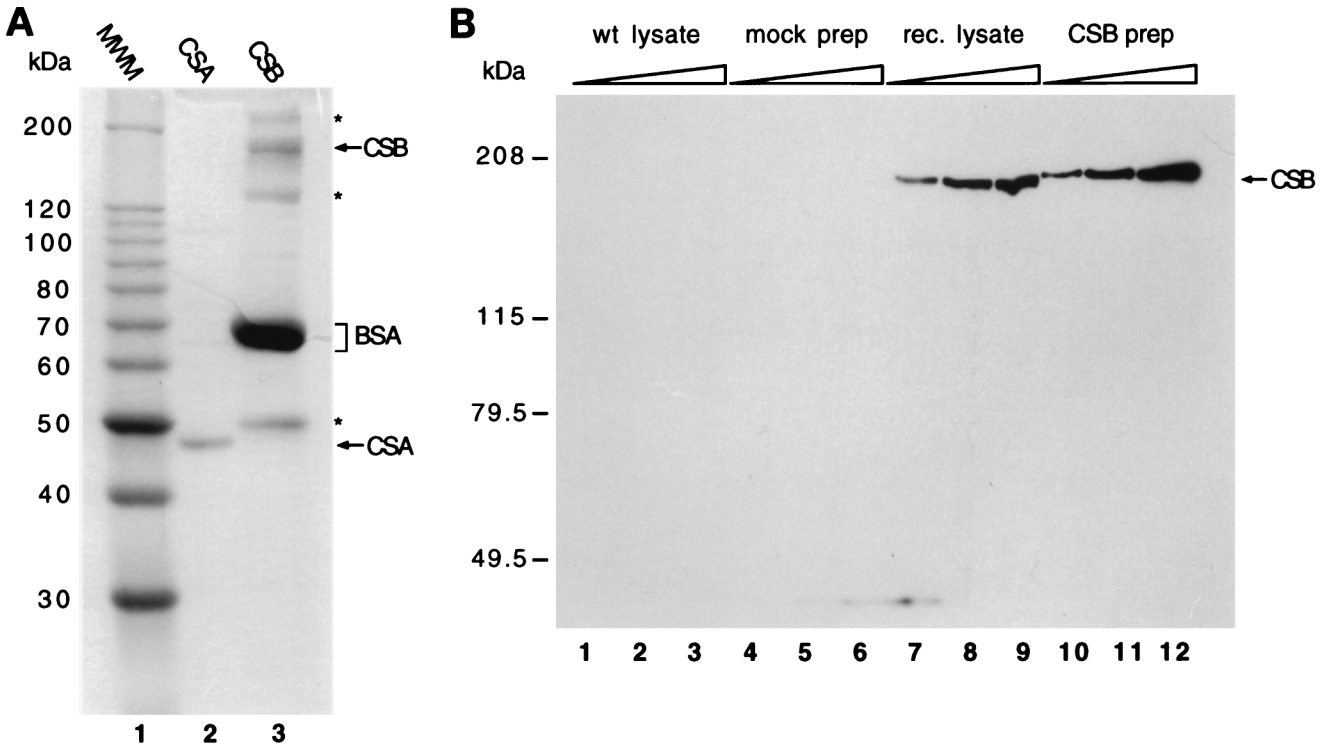



FIG. 1. (A) SDS–8% polyacrylamide gel containing molecular mass standards (MWM) (lane 1), bacterially expressed CSA protein (400 ng) (lane 2), and CSB protein expressed from baculovirus (800 ng) (lane 3). CSB was stabilized with 0.2 μg of BSA per μl (bracket). The preparation used in these experiments contained three major contaminants (asterisks). (B) Western blot with anti-EK epitope monoclonal antibodies. Increasing amounts of cell lysate, corresponding to 1, 2, and 4 μg (lanes 1 to 3, respectively, and 7 to 9, respectively) or of normal and mock preparations (prep) of CSB, corresponding to 150, 300, and 600 ng (lanes 4 to 6, respectively, and 10 to 12, respectively) were resolved on an SDS–6% polyacrylamide gel, and Western blotted. wt, wild type; rec., recombinant.

preparation, the reaction mixture was incubated at room temperature for 2 minutes (except for the template in Fig. 5B, which was damaged for only 1 min), and the reaction was quenched by the addition of 6 μl of βME. The damaged and undamaged annealed probes were then ethanol precipitated, washed, and resuspended in 50 μl of TE buffer (20 mM Tris-Cl [pH 7.4], 1 mM EDTA). The unlabeled annealed probes were used directly. For the labeled probes, 5.5 μl of 10× native DNA loading buffer was added to the probes, and the mixture was loaded onto a native 15% polyacrylamide gel containing 1× TBE (90 mM Tris base [pH 8.3], 90 mM boric acid, 1 mM EDTA) and electrophoresed at 80 V for 6 h. The annealed bands, as visualized by staining with ethidium bromide and visualization under UV light, migrated with a mobility between those of the two isolated oligonucleotides. Annealing efficiency was nearly 100%. These bands were excised from the gel and soak-eluted in 800 μl of elution buffer (25 mM Tris-Cl [pH 7.9], 1 mM EDTA, 300 mM sodium acetate, 0.5% SDS) for 8 h. The elutions were split into two 400-μl samples, extracted once with an equal volume of phenol and once with phenol-chloroform-isoamyl alcohol (25:24:1), and ethanol precipitated. The pellets were resuspended in 50 μl of TE buffer.

**ATPase assay.** Reaction mixtures (20 μl) contained 30 mM Tris-Cl (pH 7.4), 0.1 M NaCl, 8 mM $MgCl_2$, 1 mM DTT, 0.5 mCi [$\gamma$-$^{32}$P]ATP, and, where indicated, 100 ng of pGEM3 plasmid DNA or 10 pmol of the GAL4staCOD oligonucleotide. The reaction mixtures were incubated at 30°C for 20 min, reactions were quenched by the addition of 2× formamide loading buffer (90% deionized formamide, 0.1% bromophenol blue and xylene cyanol), and 4 μl was resolved on a 12% polyacrylamide gel containing 7 M urea for 1.5 h at 100 V. The results were quantitated with a Molecular Dynamics PhosphorImager and ImageQuant software.

**Labeled-DNA-based gel mobility shift assay.** The 20-μl reaction mixtures contained transcription buffer, 8.25 mM $MgCl_2$, 250 μg of BSA, 5 U of RNase inhibitor, 50 ng of poly(dI · dC), 0.05% Nonidet P-40, approximately 0.25 pmol of $^{32}$P-labeled C-tailed template-probe, and where indicated, 200 ng of Pol II and ribonucleotides at 250 μM. For the $^{32}$P-labeled-DNA-based gel mobility shift assays shown in Fig. 2, the reaction mixtures were incubated for 40 min at 30°C and loaded directly onto 20-cm-long, 0.8-mm-thick 4.0% polyacrylamide gels (monoacrylamide/bisacrylamide ratio, 39:1), and electrophoresis was carried out at room temperature for 2.5 h at 120 V in a running buffer containing 0.5× TBE with 1% glycerol.

**Labeled-RNA-based gel mobility shift and transcription assays.** Conditions were identical to those for the gel mobility shift assay except that the UTP concentration was reduced to 10 μM, 0.4 μCi of [$\alpha$-$^{32}$P]UTP was included in the reaction mixtures, and approximately twice as much unlabeled template-probe was used. For the reactions shown in Fig. 2C, lane 7, and in Fig. 5B, lanes 2 and 3, 1 μM UTP replaced the usual 10 μM UTP in order to induce pausing at the H3.3 sequence. ATPγS (adenosine 5′-*O*-thiotriphosphate) (Gibco BRL) and AMP-PNP (adenyl-5′-yl imidodiphosphate) (Sigma) were substituted for ATP at the same concentrations where indicated. CSB, where added, was always present in the initial incubation. After 20 min, the appropriate antibodies, if any, were added, and 10 μl of the reaction mixture was resolved on gels similar to those described above containing 1% glycerol, 0 to 4 mM $MgCl_2$ as described in the figure legends, and 0.5× TBE. Electrophoresis was carried out at 4°C for 3 h at 150 V in a running buffer containing 0.5× TBE with 1% glycerol. For the transcription assay shown in Fig. 2B, 50 μl of stop buffer (300 mM sodium acetate, 0.2% SDS, 10 mM EDTA, 5 ng of tRNA per μl) and 10 μg of proteinase K were added to the reaction mixtures after 40 min, and the reaction mixtures were incubated at 55°C for 20 min. The reaction mixtures were extracted once with phenol and once with phenol-chloroform-isoamyl alcohol and ethanol precipitated. The labeled RNA was resolved by using a 12% polyacrylamide–urea gel, electrophoresed for 1.5 h at 300 V, and visualized by autoradiography.

**Isolation and resolution of labeled RNA from native gels.** Complexes were excised from the gel by alignment with an autoradiograph. The RNA was soak-eluted for 6 h at room temperature in elution buffer plus 0.1 mg of proteinase K per ml. The supernatant was extracted twice with phenol-chloroform-isoamyl alcohol, and the RNA was precipitated with ethanol. The RNA was resolved by electrophoresis through a 15% polyacrylamide gel containing 1× TBE and 7.0 M urea and visualized by autoradiography.

## RESULTS

**Preparation of recombinant CSA and CSB.** Figure 1A shows a Coomassie blue-stained SDS–8% polyacrylamide gel of the recombinant CSA and CSB proteins used in these experiments. CSA and CSB were expressed as $His_6$ fusion proteins and purified by nickel-chelate affinity chromatography as described in Materials and Methods. CSA was expressed and purified from *E. coli* (Fig. 1A, lane 2), and CSB was from Sf9 cells infected with recombinant baculovirus (lane 3). The CSA

bacterial expression vector was a kind gift of Errol Friedberg. The CSB cDNA, a kind gift of Jan Hoeijmakers, was subcloned into the baculovirus expression vector pBLUEBACHisA (Invitrogen) as described in Materials and Methods. CSB was stabilized with 0.2 μg of BSA per μl. We found that upon dialysis of the most concentrated CSB fractions, the protein precipitated from solution. CSB was therefore concentrated and desalted by ultrafiltration. CSA was relatively homogeneous, while the preparations of CSB used in these experiments appeared to contain three major contaminants. We also verified that the approximately 170-kDa purified protein was actually CSB by use of an EK epitope tag engineered into the expression vector. Figure 1B shows a Western blot of increasing amounts of a lysate from wild-type-baculovirus-infected Sf9 cells (lanes 1 to 3), mock-purified protein (lanes 4 to 6), a lysate from recombinant-virus-infected cells (lanes 7 to 9), or the CSB preparation shown in Fig. 1A (lanes 10 to 12). The blot was probed with anti-EK monoclonal antibodies (Invitrogen) and visualized by using the ECL system (Amersham). The antibodies specifically recognize only those preparations that are predicted to contain the CSB protein.

**Experimental system.** In *E. coli*, the TRCF encoded by the *mfd* gene has been shown to interact with bacterial RNA polymerase stalled at a DNA lesion. TRCF then dislodges the polymerase and is believed to recruit the UvrABC NER proteins (50). We could not detect a strong interaction between either CSA or CSB and human Pol II preparations in solution (data not shown), and we therefore sought to determine whether CSA and CSB could interact with actively transcribing Pol II.

Figure 2A shows a diagram of the template-probe designed for these experiments. The double-stranded DNA molecule was constructed by hybridization of two oligonucleotides as described in Materials and Methods, and it functions in both in vitro transcription assays and electrophoretic mobility shift assays (EMSAs). The template contains four features. A novel oligo(dC) tail originally developed by Kadesch and Chamberlin (20) allows Pol II to initiate and elongate in a unidirectional fashion, from left to right as shown in Fig. 2A. This manipulation circumvents the requirement for general transcription factors to specifically initiate Pol II transcription. The template also contains a GAL4 consensus element for binding of the yeast GAL4 protein and its derivatives, which were used in some control reactions. Finally, to determine whether the position or manner of Pol II stalling could influence any potential interactions with CSA or CSB, we incorporated two other novel features: a Pol II pause or stall sequence taken from the histone H3.3 gene, which causes Pol II to pause at low UTP concentrations (21), and a 3-nucleotide heteroduplex region, positioned 20 bp from the end of the duplex, which contains six mispaired thymidine residues that are hypersensitive to oxidation by permanganate. Mild oxidation by permanganate yields predominantly thymine glycols (11), nonbulky lesions that can be recognized by the NER machinery (24) and other repair mechanisms that are susceptible to TCR and defective in CS (7, 25, 27). Pol II is expected to stall at the oxidized residues.

Transcription assays performed with this template and a highly purified Pol II preparation produce a runoff transcript of approximately 72 nucleotides, corresponding to the length of the duplex region (Fig. 2B, lane 2). Oxidation by permanganate (Fig. 2C, lanes 4 to 6) or reduction of the UTP concentration (lane 7) generates products of the size predicted if Pol II had stalled at the lesion or the stall sequence. At this level of oxidation, approximately 50% of the products pause prematurely; higher levels of damage caused increased stalling at cryptic sites. For consistency, all subsequent experiments employed a template that was damaged with this level of permanganate, although we found that the position and manner of stalling or the presence of the stall sequence had no effect under our assay conditions (see below).

In addition to labeling RNA runoff transcripts with [$\alpha$-$^{32}$P]UTP, we also $^{32}$P end labeled one strand of the template directly with [$\gamma$-$^{32}$P]ATP and polynucleotide kinase to study the assembly of complexes on the template by gel mobility shift assays. An immunopurified and highly homogeneous preparation of Pol II interacted with the labeled probe, producing two prominent shifts with different mobilities (Fig. 3A, lane 1). The result is the same in the absence of nucleotides (Fig. 3A, lane 1), in the presence of 250 μM ATP or nucleoside triphosphates (NTPs) (lanes 4 and 7), or in the absence of DNA damage (data not shown). In the presence of a preparation of TFIIH, which has a potent Pol II carboxy-terminal domain (CTD) kinase activity (reviewed in reference 15) and either ATP or NTPs (Fig. 3A, lanes 6 and 9), all of the mobility shift signal is converted to the upper form. We found that during the purification of Pol II, early elutions from the antibody matrix contained predominantly the Pol IIA form, and later elutions contained the Pol IIO form (see Materials and Methods). This may reflect a greater affinity of this antibody preparation for Pol IIO, an effect which was also evident in Western blots and gel mobility shift experiments (see, for example, Fig. 3D and 4B). We were thus able to correlate the upper band to the hyperphosphorylated form and the lower band to the hypophosphorylated form of Pol II (Fig. 3B, lanes 2 to 4). All other experiments were performed with a preparation of Pol II that contains approximately equal amounts of Pol IIA and Pol IIO.

To confirm that Pol II and the damaged template produced a Pol II-DNA-RNA ternary complex in the presence of NTPs, we used [$\alpha$-$^{32}$P]UTP and an unlabeled template in a $^{32}$P-labeled-RNA-based gel mobility shift assay. We observed bands identical to those in the labeled-DNA-based gel mobility shift assay (Fig. 3C, lane 1) that could be chased to free RNA with the addition of 0.5 mg of proteinase K per ml (lane 2). We conclude that the complex is indeed a ternary complex containing RNA, DNA, and Pol II.

In addition to the EMSA data, Western blotting revealed that the largest Pol II subunit was converted by TFIIH and ATP to a higher-molecular-weight form in a reaction blocked by the H8 kinase inhibitor. Figure 3D shows a Western blot of an SDS–6% polyacrylamide gel with monoclonal antibodies against the Pol II CTD (8WG16) (53). Pol II-containing reactions were performed under conditions identical to those for the gel shift assay and contained TFIIH (Fig. 3D, lanes 2 to 4), ATP (lanes 3 and 4), and the kinase inhibitor H8 (lane 4). Only when TFIIH and ATP were present in the absence of the kinase inhibitor did the anti-CTD antibody detect the higher-molecular-weight hyperphosphorylated IIO form of the Pol II large subunit (Fig. 3D, lane 3).

A similar result was observed with an EMSA in the presence of all four nucleotides (Fig. 3E). TFIIH can phosphorylate Pol II to a low degree in the presence of only GTP, CTP, and UTP (Fig. 3E, lane 2); however, in the presence of ATP, phosphorylation is increased (lane 3). When the H8 kinase inhibitor was added to the reaction mixture, phosphorylation was completely blocked, analogous to the situation in Fig. 3D (lane 4). TFIIH did not, however, interact with the probe or supershift Pol II above the IIO form in the EMSA under conditions where Pol II is known to be transcribing and stalling at the damage site and the end of the template, indicating that TFIIH alone does not interact with such complexes (Fig. 3E, lane 3; also Fig. 3A, lane 9). This is in agreement with the results of others (63) and indicates that TFIIH is not the eukaryotic equivalent of TRCF.

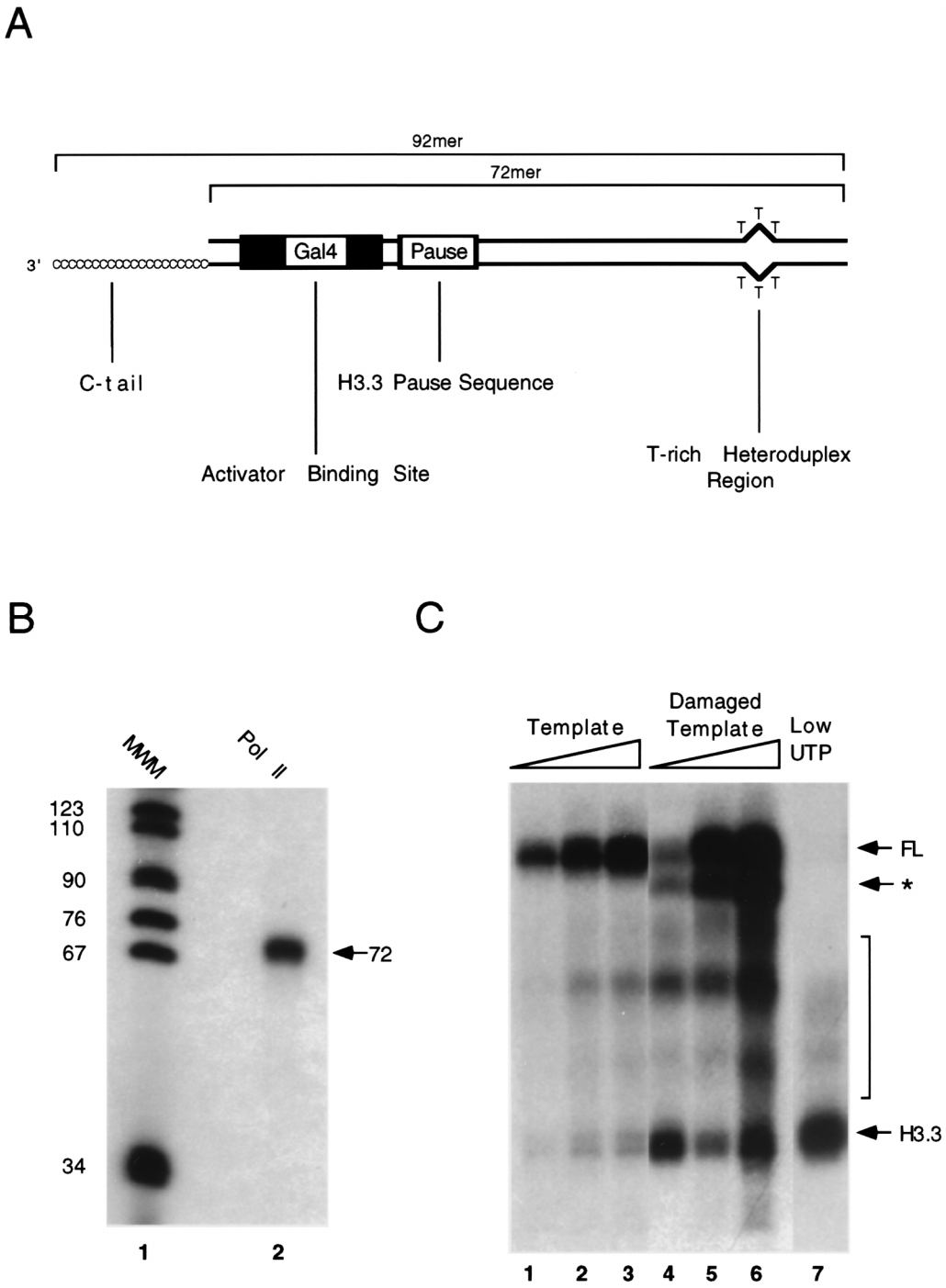



FIG. 2. (A) The GAL4sta template-probe. The molecule contains a single-stranded C tail for initiation by Pol II, a consensus GAL4 binding site, a pause sequence taken from the histone H3.3 gene, and a small T-rich heteroduplex region that can be damaged by $KMnO_4$. The lower strand is the oligonucleotide GAL4staTEM; the upper strand is GAL4staCOD. Sequences are given in Materials and Methods. (B) Runoff transcription with the template shown in panel A and 200 ng of immunopurified Pol II. The RNA transcript corresponds to the length of the duplex region. MWM, molecular weight markers (in thousands). (C) Runoff transcriptions produced by using damaged templates mildly oxidized by $KMnO_4$. Increasing amounts of either undamaged template DNA or template DNA that had been damaged with $KMnO_4$ as described in Materials and Methods (0.2, 0.4, and 0.8 pmol) were used under assay conditions identical to those for panel B. Damaging the template causes over 50% of the transcripts to stop prematurely. FL, full-length transcripts. Transcripts truncated by the presence of damage at the heteroduplex are denoted by the asterisk. Transcripts blocked at the H3.3 pause sequence are labeled H3.3. In lane 7 the usual 10 μM unlabeled UTP was replaced with 1 μM UTP. Cryptic stall sequences are bracketed.

**CSB interacts with Pol II bound to and transcribing DNA.** Initially we added recombinant CSB protein to stalled Pol II in labeled-DNA-based gel mobility shift assays under conditions in which Pol II was known to be transcribing and stalling at the heteroduplex region. CSB appeared to bind directly to the probe (data not shown), in agreement with the results of Selby and Sancar (49). When this same reaction was resolved with gels containing $MgCl_2$, however, the direct interaction largely

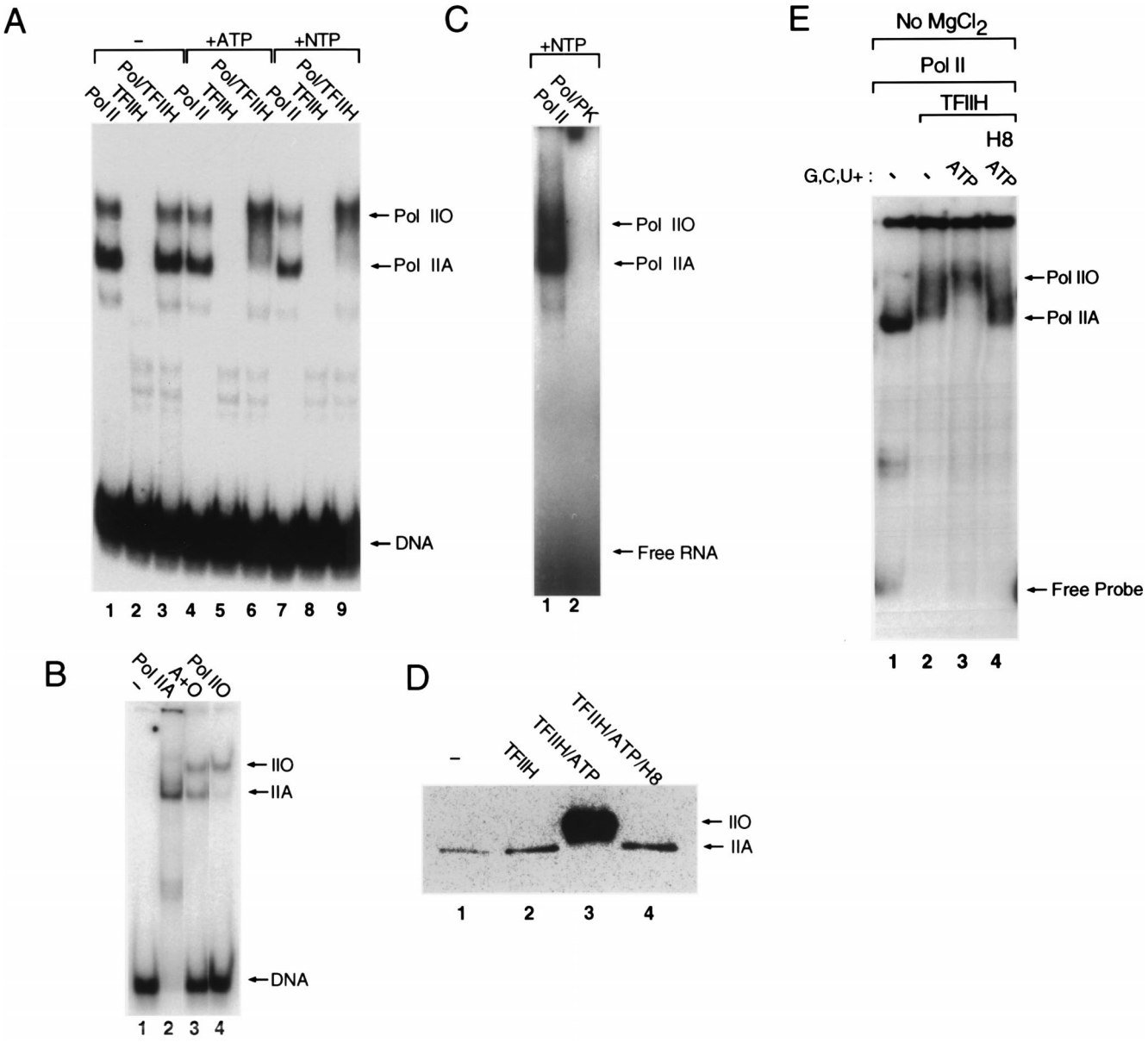



FIG. 3. (A) EMSA employing purified Pol II, TFIIH, and various nucleotide combinations. The 4.0% polyacrylamide gel contained 0.5× TBE with 1% glycerol, as did the running buffer. Two hundred nanograms of Pol II creates two mobility shifts, whereas 150 ng of TFIIH does not bind the probe to a significant extent (lanes 1 and 2, respectively). Inclusion of 250 μM ATP or NTPs results in a conversion of the Pol II bands from a higher- to the lower-mobility form in the presence of TFIIH (lanes 6 and 9) but does not result in an appreciable supershift. (B) The two different mobility shifts seen with Pol II correspond to the two different phosphorylation states, Pol IIA and Pol IIO. Assays were performed identically to those for panel A and included the labeled probe (lane 1) and Pol II preparations that had been shown via silver staining (data not shown) to contain predominantly the Pol IIA form (lane 2), a mixture of the Pol IIA and Pol IIO forms (lane 3), or predominantly the Pol IIO form (lane 4). (C) RNA gel mobility shift assay was conducted under conditions identical to those for panels A and B, except that the probe was unlabeled and present in approximately twofold greater amounts, the UTP concentration was lowered to 10 μM, and 0.2 μCi of [α-$^{32}$P]UTP was included in the reaction. In lane 2, 0.5 μg of proteinase K (PK) per ml was added to the reaction mixture after 15 min. Degradation of the labeled-RNA-containing signal was minimized by shorter incubation times and electrophoresis at 4°C (see Materials and Methods). (D) Western blot with monoclonal antibodies against the Pol II CTD. Reaction conditions were identical to EMSA conditions except that no probe was included. The reaction mixtures contained Pol IIA (lane 1) plus TFIIH (lane 2) or TFIIH and 250 μM ATP (lanes 3 and 4). Lane 4 contained the kinase inhibitor H8 at 1 mM. The reactions were stopped by the addition of SDS-polyacrylamide gel electrophoresis loading buffer, and the mixtures were briefly heated to 90°C, resolved on an SDS–6% polyacrylamide gel, and Western blotted with the 8WG16 monoclonal antibody. (E) Labeled-DNA-based gel mobility shift assay demonstrating phosphorylation of the Pol II CSB by TFIIH in a manner counteracted by H8. The same amounts of proteins were used. Note that GTP, CTP, and UTP can partly substitute for ATP as a substrate for the TFIIH kinase.

disappeared (unpublished observations). We therefore used gel shift conditions that included $MgCl_2$ in all subsequent experiments.

In standard labeled-DNA-based gel mobility shift assays containing $MgCl_2$, it appeared that the CSB protein could interact with Pol II but not with GAL4-VP16 and not directly with the probe in the absence of added protein (data not shown). To demonstrate an interaction between CSB and Pol II and to demonstrate that the mobility shifts contained RNA and an actively transcribing polymerase, we performed $^{32}$P-labeled-RNA-based mobility shift assays (Fig. 4A to C) similar to those described for Fig. 3C. We found that inclusion of CSB

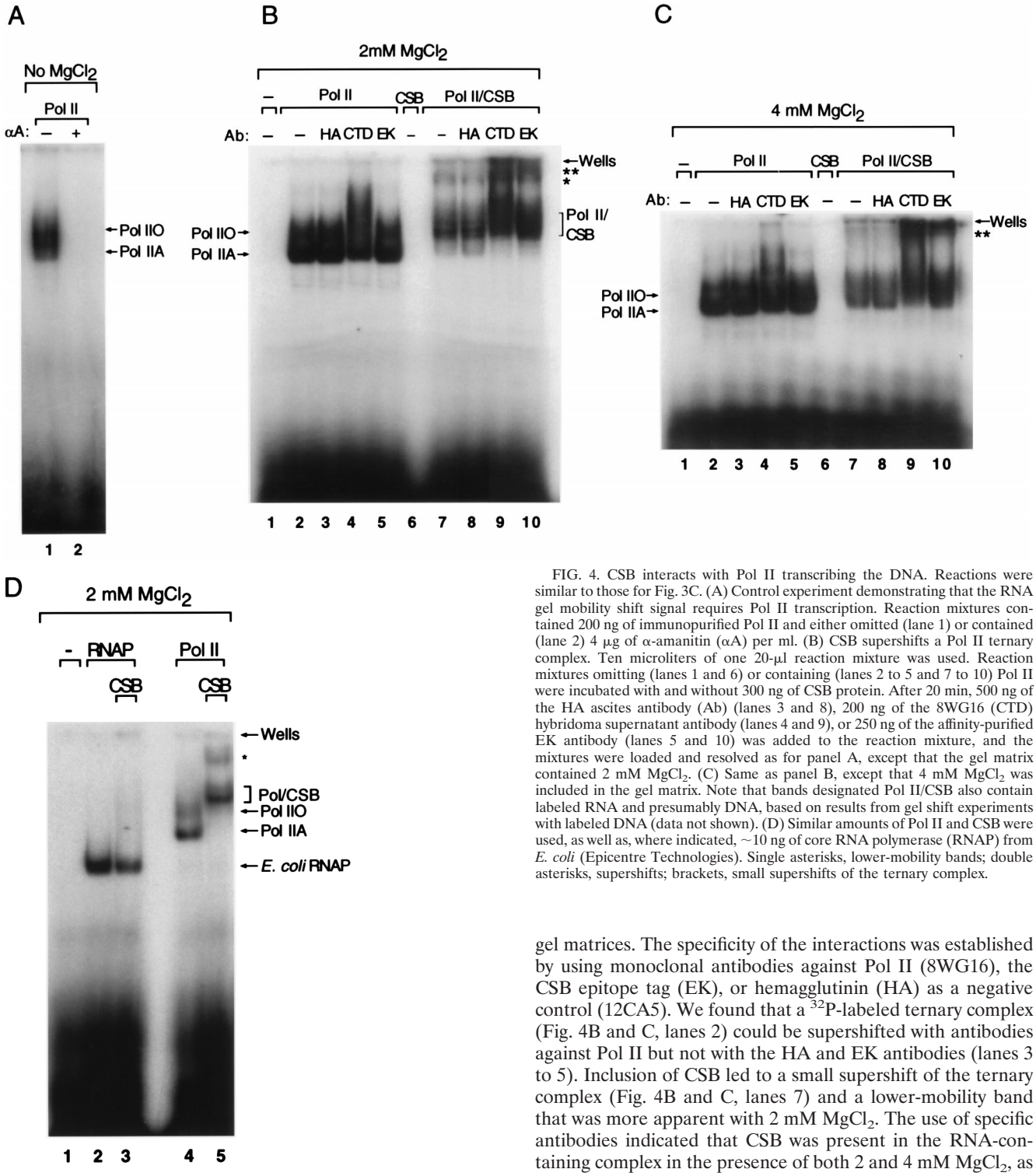



FIG. 4. CSB interacts with Pol II transcribing the DNA. Reactions were similar to those for Fig. 3C. (A) Control experiment demonstrating that the RNA gel mobility shift signal requires Pol II transcription. Reaction mixtures contained 200 ng of immunopurified Pol II and either omitted (lane 1) or contained (lane 2) 4 μg of α-amanitin (αA) per ml. (B) CSB supershifts a Pol II ternary complex. Ten microliters of one 20-μl reaction mixture was used. Reaction mixtures omitting (lanes 1 and 6) or containing (lanes 2 to 5 and 7 to 10) Pol II were incubated with and without 300 ng of CSB protein. After 20 min, 500 ng of the HA ascites antibody (Ab) (lanes 3 and 8), 200 ng of the 8WG16 (CTD) hybridoma supernatant antibody (lanes 4 and 9), or 250 ng of the affinity-purified EK antibody (lanes 5 and 10) was added to the reaction mixture, and the mixtures were loaded and resolved as for panel A, except that the gel matrix contained 2 mM $MgCl_2$. (C) Same as panel B, except that 4 mM $MgCl_2$ was included in the gel matrix. Note that bands designated Pol II/CSB also contain labeled RNA and presumably DNA, based on results from gel shift experiments with labeled DNA (data not shown). (D) Similar amounts of Pol II and CSB were used, as well as, where indicated, ~10 ng of core RNA polymerase (RNAP) from *E. coli* (Epicentre Technologies). Single asterisks, lower-mobility bands; double asterisks, supershifts; brackets, small supershifts of the ternary complex.

led to a moderate supershift of the Pol II ternary complex. Figure 4A shows a control experiment demonstrating that Pol II elongation is necessary for the signal. The RNA mobility shift pattern was eliminated in the presence of 4 μg of α-amanitin per ml (Fig. 4A, lane 2). In Fig. 4B and C, halves of the reaction mixtures were loaded onto two gels that were identical except for the presence of 2 and 4 mM $MgCl_2$ in the gel matrices. The specificity of the interactions was established by using monoclonal antibodies against Pol II (8WG16), the CSB epitope tag (EK), or hemagglutinin (HA) as a negative control (12CA5). We found that a $^{32}P$-labeled ternary complex (Fig. 4B and C, lanes 2) could be supershifted with antibodies against Pol II but not with the HA and EK antibodies (lanes 3 to 5). Inclusion of CSB led to a small supershift of the ternary complex (Fig. 4B and C, lanes 7) and a lower-mobility band that was more apparent with 2 mM $MgCl_2$. The use of specific antibodies indicated that CSB was present in the RNA-containing complex in the presence of both 2 and 4 mM $MgCl_2$, as the CTD and EK antibodies generated a supershift (Fig. 4B and C, lanes 9 and 10) but the HA antibody had no effect (lanes 8). CSA did not score in this assay (data not shown).

As a further measure of specificity, we employed the same assay with ternary complexes containing the *E. coli* core ($\alpha_2\beta\beta'$) RNA polymerase. Figure 4D shows that, unlike the case for Pol II (lane 5), little interaction occurs between the *E. coli* polymerase ternary complex and CSB (lane 3).

To test against the possibility that cryptic stalling at the H3.3

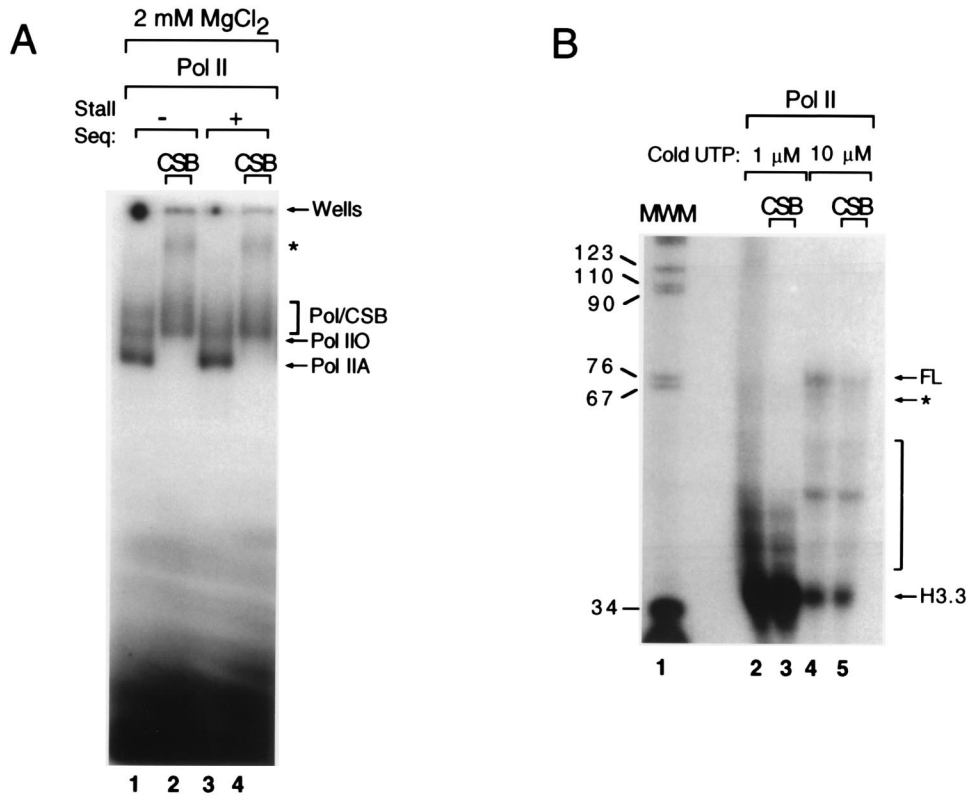
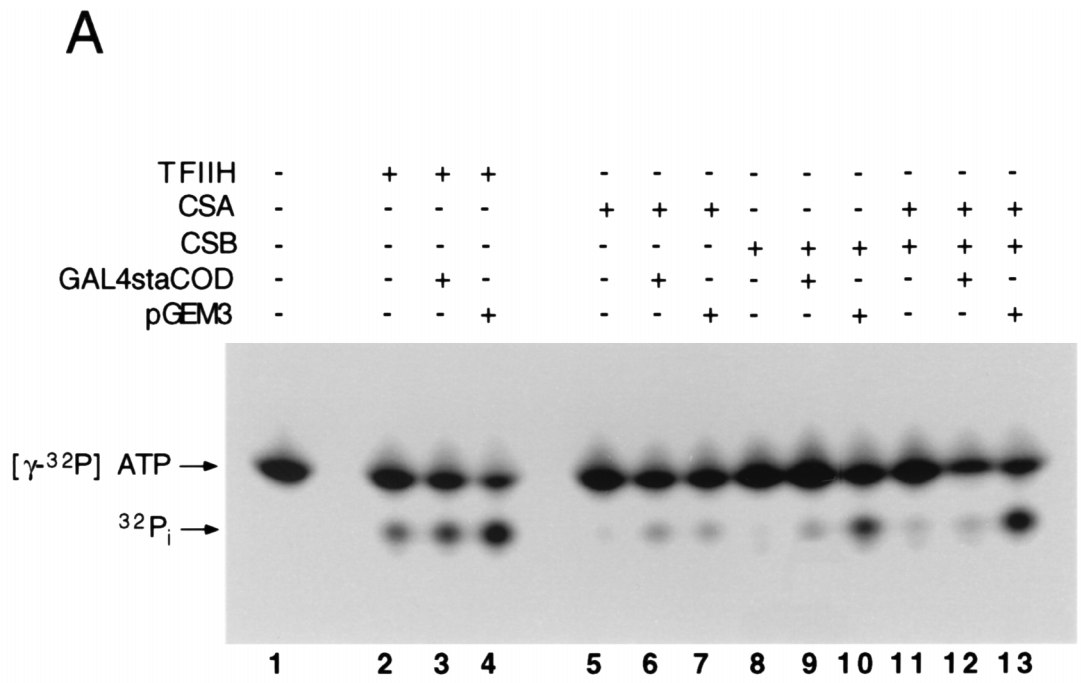



FIG. 5. The position and manner of Pol II stalling do not influence the CSB interaction with the ternary complex. (A) The reaction conditions and amounts of proteins were identical to those in Fig. 4. Pol II ternary complexes formed on templates either lacking (lane 1) or containing (lane 3) the H3.3 stall sequence (Stall Seq) were equally supershifted by the CSB protein (lanes 2 and 4). The presence and position of the low-mobility band (asterisk) were also unaltered. (B) Complexes resolved by using a labeled-RNA-based gel mobility shift assay similar to that in panel A were excised from the gel, and the RNA was purified as described in Materials and Methods. Under either stalling (low UTP; lanes 2 and 3) or nonstalling (high UTP; lanes 4 and 5) conditions, the species of labeled RNA recovered were the same whether purified from Pol II ternary complexes (lanes 2 and 4) or CSB-supershifted complexes (lanes 3 and 5). FL, full-length RNA. RNA prematurely truncated due to damage at the heteroduplex is shown with the asterisk. The stall sequence is denoted H3.3. Cryptic stall sequences are shown by the brackets. An autoradiograph of a 15% polyacrylamide–urea gel is shown. MWM, molecular weight markers (in thousands).

stall sequence was responsible for the CSB interactions, even at high UTP concentrations, or that CSB interacted with only a subset of stalled elongation complexes, we removed the H3.3 sequence from the template, replaced it with a sequence from a G-less cassette template, and analyzed the composition of the labeled RNA in the native gel complexes. Figure 5A shows a labeled-RNA-based gel mobility shift assay identical to those in Fig. 4. As is the case with the template containing the stall sequence (Fig. 5A, lanes 3 and 4), a Pol II ternary complex formed on a template in which the stall sequence is removed is still supershifted by CSB (lanes 1 and 2). Figure 5B shows an assay similar to the transcription assay shown in Fig. 2C, but with two major differences. First, the reactions were resolved by using a native acrylamide gel, similar to those for Fig. 4, the protein complexes (either the Pol II complex or the CSB supershifted complex) were excised and eluted from the gel matrix, and the RNA was resolved on a denaturing gel. Second, the template was damaged with the same level of permanganate, but for only half the normal time. In the presence or absence of high concentrations of UTP, RNA of the same molecular weight distribution was recovered from the CSB-supershifted complex (Fig. 5B, lanes 3 and 5) and with Pol II alone (lanes 2 and 4). We conclude that the CSB interaction is independent of the position and manner of Pol II stalling under the conditions employed in these assays.

**A stable interaction between CSB and the Pol II ternary complex requires a hydrolyzable ATP β-γ phosphoanhydride bond.** We tested the CSA and CSB preparations separately and together in ATPase assays with and without double-stranded plasmid (pGEM3) and single-stranded oligonucleotide (GAL4staCOD) DNA. Figure 6A shows an ATPase assay in which TFIIH, CSA, and CSB were incubated with [γ-$^{32}$P]ATP. Intact ATP and free labeled phosphate ($P_i$) were resolved on a 12% polyacrylamide–urea gel, and the ratio was quantitated by PhosphorImager analysis. The ratio of the bottom to the top bands provides a relative measure of ATPase activity. Compared to the TFIIH control (Fig. 6A, lanes 2 to 4), CSA had little ATPase activity under any assay conditions (lanes 5 to 7). CSB had little intrinsic ATPase activity under these conditions (Fig. 6A, lane 8), but a baseline ATPase activity was apparent with longer incubations (data not shown). The ATPase activity was induced by single-stranded and, somewhat more strongly, double-stranded DNAs (lanes 9 and 10). These results are in agreement with those in reference 49, although the effect seen in this study was greater with single-stranded M13 DNA. The addition of tRNA had no effect (unpublished observations). Interestingly, we also found that the addition of CSA resulted in a moderate (approximately twofold) stimulation of CSB's ATPase activity (Fig. 6A, lanes 11 to 13). These results are quantitated in Fig. 6B. The ATPase activity was due to CSB and not a contaminant, as the results of an ATPase assay performed with CSB immunoprecipitated from purified fractions with anti-EK, but not mock antibodies, were identical to the results shown in Fig. 6A (data not shown).



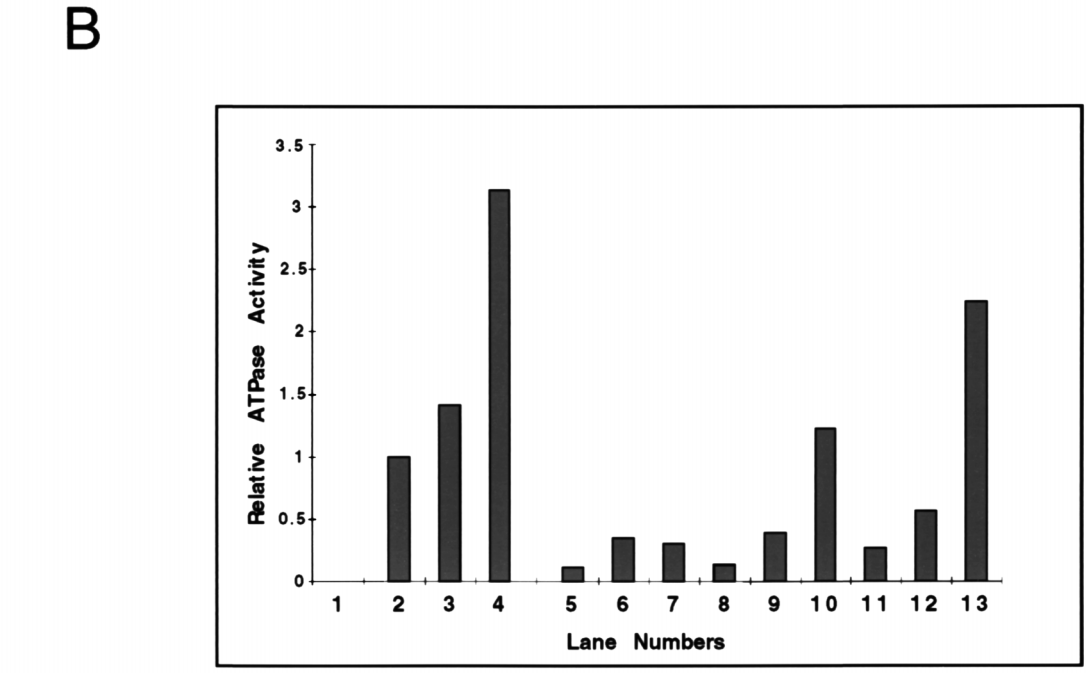




FIG. 6. (A) CSB contains an ATPase activity that is stimulated by DNA. [γ-$^{32}$P]ATP (0.5 μCi) (lane 1) was incubated with 150 ng of TFIIH (lanes 2 to 4), 40 ng of CSA (lanes 5 to 7), or 300 ng of CSB (lanes 8 to 10). The reaction mixtures contained either no added DNA (lanes 2, 5, and 8), 10 pmol of single-stranded DNA (GAL4staCOD) (lanes 3, 6, and 9), or 100 ng of double-stranded plasmid DNA (pGEM3) (lanes 4, 7, and 10). One hundred fifty nanograms of TFIIH purified through five chromatographic steps (52a) is saturating in reconstituted in vitro transcription reaction mixtures of equal volume (unpublished data). (B) Quantitation of the results shown in panel A. Quantitation was performed with ImageQuant software (Molecular Dynamics). The amount of hydrolysis by TFIIH alone was taken as 1 U of ATPase activity.

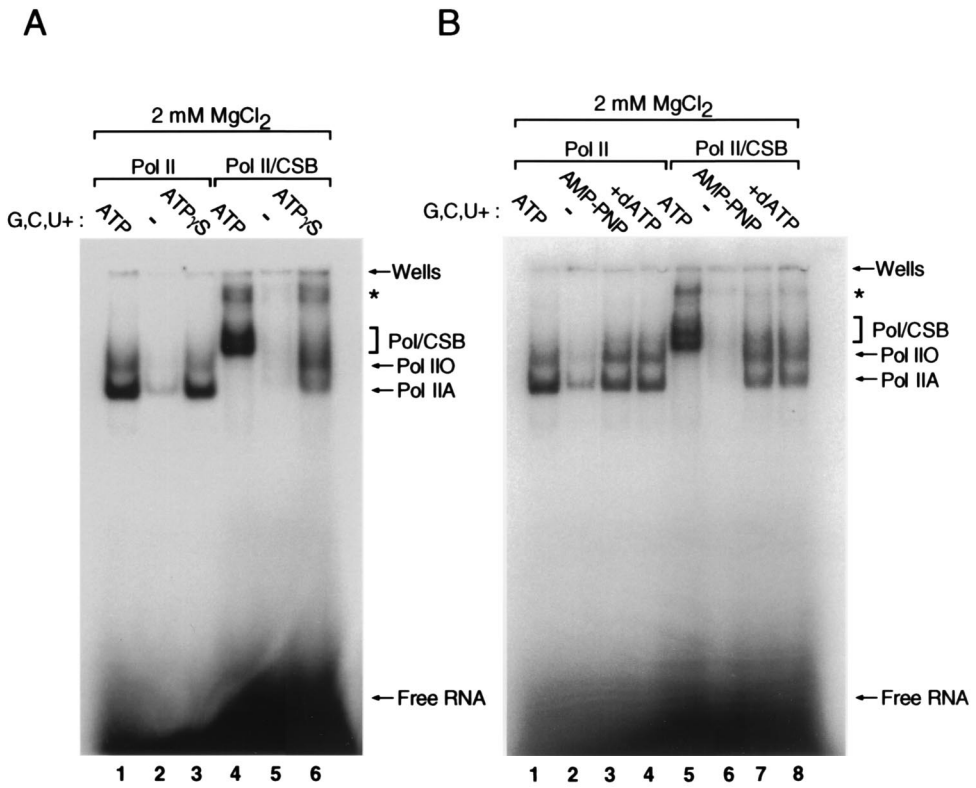



FIG. 7. A hydrolyzable ATP β-γ bond is involved in the CSB-Pol II ternary complex interaction. Both gels contained 2 mM $MgCl_2$ in the matrix. (A) RNA mobility shift assays included 250 μM GTP and CTP, 10 μM UTP, 0.2 μCi of [α-$^{32}$P]UTP, and either 250 μM ATP (lanes 1 and 4), no added ATP (lanes 2 and 5), or 250 mM ATPγS (lanes 3 and 6). (B) dATP cannot substitute for ATP in providing a hydrolyzable β-γ bond. Reactions were identical to those for panel A except that AMP-PNP rather than ATPγS was included and, where indicated, 250 μM dATP was added to the reaction mixtures. Note that bands designated Pol II/CSB also contain labeled RNA and presumably DNA. Brackets, supershift complex; asterisks, lower-mobility bands.

By performing experiments similar to those shown in Fig. 4B and C in the presence of the poorly hydrolyzable ATP analogs ATPγS and AMP-PNP (Fig. 7), we found that the CSB-Pol II ternary complex interaction requires a hydrolyzable ATP β-γ phosphoanhydride bond. Under transcription assay conditions, the C-tailed template is transcribed equally well by Pol II in the presence of ATP, ATPγS, or AMP-PNP (data not shown) and produces equivalent amounts of labeled ternary complexes in labeled-RNA-based gel mobility shift assays (compare lanes 1 and 3 in Fig. 7). In the absence of any adenine nucleotide, little transcription occurred (Fig. 7, lanes 2). When CSB was added to the ternary complex, a typical supershift complex was observed (Fig. 7A, lane 4, and 7B, lane 5), as well as the lower-mobility band. When ATP was replaced with ATPγS or AMP-PNP, we found that the supershifted complex failed to form efficiently (compare lane 6 with lane 4 in Fig. 7A and lane 7 in with lane 5 in Fig. 7B). The hypophosphorylated form of Pol II appeared to be completely unaffected by CSB, whereas the hyperphosphorylated form was still shifted upward slightly. The low-mobility band was unaffected by the replacement of ATP with ATPγS and was reduced in the presence of AMP-PNP (Fig. 7A), indicating that it is either (i) a nonspecific interaction or (ii) a specific interaction that is not dependent on a hydrolyzable ATP β-γ bond. We conclude that most of the CSB interaction with the Pol II-DNA-RNA ternary complex requires ATP β-γ bond hydrolysis. In the case of AMP-PNP, we supplemented the reaction mixtures with dATP to see if it could satisfy the energy requirement. We found that AMP-PNP plus dATP could not substitute for ATP in this assay (Fig. 7B, lane 8).

## DISCUSSION

Our data show that CSB can bind in vitro to an elongating Pol II molecule in a highly purified transcription system lacking the general transcription factors. We argue that the CSB-containing Pol II ternary complex is likely to be an intermediary for a complex involved in the higher rates of NER in active genes. The subsequent complex is expected to contain TFIIH or a related factor, as this would explain all available genetic and biochemical information, including the observation that mutations in CSA and CSB, as well as specific mutations in a subset of TFIIH subunits, can cause CS and the finding that CSB binds to TFIIH and TFIIH-associated factors in vitro (19, 49).

We found that CSB is an ATPase (Fig. 6), in agreement with the results of others (49). However, we observed that CSB's ATPase activity was more strongly activated by double-stranded DNA than by single-stranded DNA. The difference in results may reflect a difference in the number of molecules or a difference in the size, structure, or composition of the single-stranded DNA. Our results also indicate that CSB's ATPase activity may be important for mediating the interaction with a Pol II-containing ternary complex (Fig. 7).

CSA and CSB are implicated in the facilitated repair of active genes both genetically and biochemically (reviewed in

reference 12). Cells taken from CS patients are defective in TCR (26, 57, 59). Our results indicate that CSB can interact with Pol II molecules under conditions which we imagine mimic the in vivo situation, where a transcribing molecule of Pol II pauses upon encountering a DNA lesion. The interactions were specific, as the complexes were supershifted by the appropriate monoclonal antibodies against Pol II and the CSB EK epitope (Fig. 4B and C). Furthermore, CSB-supershifted ternary complexes did not form in labeled-DNA-based gel mobility shift assays either without bound Pol II (data not shown), with the GAL4-VP16 protein bound to the template as a negative control (data not shown), or with *E. coli* RNA polymerase (Fig. 4D). The CSB interaction with the $^{32}$P-labeled ternary complex required ATP β-γ bond hydrolysis and could not be rescued by dATP (Fig. 7). Similar cases of ATP β-γ bond hydrolysis requirements have been described for other interactions involving protein, DNA, and RNA. MOT1, which contains helicase domains similar to that of CSB, appears to require ATP hydrolysis to interact with TATA-binding protein, concurrently displacing it from DNA (2). Binding of the origin recognition complex to replication origins has recently been shown to be ATP hydrolysis dependent (22). Finally, it appears that formation of a spliceosome structure requires several ATP-dependent steps which may be mediated by a class of RNA helicases known as PRPs (reviewed in reference 35).

Our ability to detect a CSB interaction with the ternary complex in the presence of nucleotides but not in solution (data not shown) may indicate either that the interaction requires a certain conformation of Pol II not presented in solution or that a strong interaction requires a properly formed complex with DNA and/or RNA. Because CSB can bind DNA directly at low $Mg^{2+}$ concentrations (data not shown) (49) and contains helicase motifs and because much of the CSB-Pol II ternary complex interaction is ATP dependent, we support the latter view, in which the interaction is stabilized by nucleic acids, either through a direct interaction or through the induction of a specific structure in an ATP-dependent fashion. In further support of this idea, we have observed weak interactions with purified labeled RNA, as well as DNA, in similar gel mobility shift assays (unpublished observations). $Mg^{2+}$ likely also plays an important role in the interactions, as we found that the presence of $MgCl_2$ in the gel matrix and running buffer could modulate the strength and possibly the specificity of the interactions (unpublished observations).

The observation that CSA had no effect on its own and was not required for the interactions described here may be explained by the fact that CSB, and not CSA, appears to contain important enzymatic activities and the finding that WD repeat-containing proteins are frequently regulatory in nature (36). TCR of oxidative base damage has been shown to be only partially defective in CSA mutant cells but fully defective in CSB and XPG/CS cells (7, 26). 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 CSB protein may have partially copurified with an endogenous insect homolog of CSA.

In order to determine whether the position or manner of Pol II stalling could influence the interaction with CSB, we introduced the heteroduplex and H3.3 stall sequence into the template. We observed identical results in the absence of damage with $KMnO_4$, under conditions in which Pol II is known to stall at the H3.3 sequence, and in the absence of the stall sequence (Fig. 5 and data not shown), indicating that the position of the polymerase, whether at the stall sequence, the damaged heteroduplex, or the end of the DNA template, did not influence the interaction with CSB. It is possible that Pol II that is paused at the end of the DNA template is also normally a target for a CSB interaction. DNA strand breaks, which are known to be a major form of damage resulting from ionizing radiation (IR), are also susceptible to TCR. Transcription coupling of IR-induced damage is defective in some CS cells, which can be IR as well as UV sensitive (26). Alternatively, other factors not included in our purified system, such as TFIIF, may serve to mask Pol II in vivo until it encounters a DNA lesion. It is also interesting that one study observed in vivo transcription elongation defects in CSB cells, proposing a mechanism whereby Pol II is defective in reading through transient pauses (3).

CSB appears to function, at least partly, through a mechanism similar to that of *E. coli* TRCF; however, the CSB protein and the *mfd* gene product have little homology. Thus, as with many other proteins involved in NER, the human and *E. coli* factors share some functional, but little structural, homology. One key difference appears to be that Pol II is stably retained after CSB binding (Fig. 4, 5, and 7), whereas in the case of TRCF, bacterial RNA polymerase is believed to be removed (50). This may reflect a relatively greater importance in eukaryotic cells of completing the transcription of genes, which are much longer than those in prokaryotes.

## ACKNOWLEDGMENTS

We thank J. Hoeijmakers for the gift of the CSB cDNA and E. Friedberg for the CSA bacterial expression vector (pTRCHisB-CSA). We thank D. Reinberg, E. Friedberg, and A. Courey for critical reading of the manuscript and J. Gralla and A. Berk for helpful advice.

This work was supported by a grant from the Margaret E. Early Trust and by grants from the National Institutes of Health (GM 46424 and GM 07185).

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