the SH2 domain. In contrast, within a monomeric SH3–SH2/tail complex, the ab loop on the SH3 domain is remote from the phosphopeptide (Fig. 1), and any contact between the two seems unlikely, given the short hinge region between the covalently adjacent domains. In particular, the intermolecular SH3/SH2 contact in our crystals could not form intramolecularly without substantial unfolding of one or both domains.

These conservations and correlations lead us to propose that the dimer structure may represent a model for the regulatory regions in a 'closed' state of Src-family kinases. In this state, the SH3 and SH2 domains and the C-terminal tails would all be sequestered and the kinase domains presumably oriented to prevent cross phosphorylation (Fig. 3*c*). Transition to an open, probably monomeric, state would free the SH3 and SH2 domains to bind heterologous ligands and to direct, together with the unique N-terminal segment, the specificity and cellular localization of the active kinase. □

Received 21 January; accepted 7 March 1994.

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ACKNOWLEDGEMENTS. We thank H. Band, L. Cantley, B. Mayer, M. Milburn and D. Winkler for discussion; H. Band for information before publication; and A. Musacchio, M. Noble, M. Saraste and R. Wierenga for atomic coordinates of the spectrin and Fyn SH3 domains. S.E.S. thanks the NSF for support. Coordinates will be deposited with the Brookhaven Protein Databank.

Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II

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THE RNA polymerase II general transcription factor TFIIH is composed of several polypeptides. The observation that the largest subunit of TFIIH is the excision-repair protein XPB/ERCC3 (ref. 1), a helicase implicated in the human DNA-repair disorders xeroderma pigmentosum (XP) and Cockayne's syndrome[2,3], suggests a functional link between transcription and DNA repair[4,5]. To understand the connection between these two cellular processes, we have extensively purified and functionally analysed TFIIH. We find that TFIIH has a dual role, being required for basal transcription of class II genes and for participation in DNA-excision repair. TFIIH is shown to complement three different cell extracts deficient in excision repair: XPB/ERCC3, XPC and XPD/ERCC2. The complementation of XPB and XPD is a consequence of ERCC3 and ERCC2 being integral subunits of TFIIH, whereas complementation of XPC is due to an association of this polypeptide with TFIIH. We found that the general transcription factor IIE negatively modulates the helicase activity of TFIIH through a direct interaction between TFIIE and the ERCC3 subunit of TFIIH.

TFIIH was purified from HeLa cell nuclear extract as shown in Fig. 1*a*. Silver staining of the transcriptionally active pool from the final purification step revealed the presence of several polypeptides (Fig. 1*d*, lane 1). Of these polypeptides, three could be correlated with proteins already described. As found previously[1], the 89K polypeptide was shown to be ERCC3 by western analysis (Fig. 1*d*, lane 2). The 62K subunit of TFIIH was detected by western blotting and silver staining[6] (Fig. 1*d*, lanes 1 and 2), as expected. Interestingly, antibodies directed against ERCC2 identified a polypeptide with an R_f value of ~80K (Fig. 1*d*, lane 2) which was not detectable by silver staining. Thus, the polypeptide composition of TFIIH appears to contain, in addition to p62, two polypeptides known to participate in excision repair, ERCC2 and ERCC3. We also identified a polypeptide of M_r 56K in the TFIIH preparation (Fig. 1*d*, asterisk). This polypeptide is the largest subunit of TFIIE[7–9] as determined by western analysis (data not shown, and see below).

To analyse the polypeptide composition of TFIIH further, we did the following experiments. First, we investigated whether ERCC2, ERCC3 and p62 coeluted with TFIIH activity during the different steps of purification; Fig. 1*a* shows the coelution on phenyl-Superose chromatography of these polypeptides with TFIIH activity. Second, we tested whether ERCC2 and/or ERCC3 could exist in a free form not associated with the TFIIH complex using quantitative western analysis; we found all ERCC3-reacting material in nuclear extract copurifying with TFIIH activity. The result was similar with ERCC2, but ~5% of ERCC2 was detected as free polypeptide upon fractionation on a gel filtration column (data not shown). Third, antibodies against ERCC2 not only immunoprecipitated ERCC2, but also ERCC3 and the 62K subunits of TFIIH (Fig. 1*b*). Finally, the ratio of ERCC2 and ERCC3 to p62 during the different steps of purification was found to be constant in the TFIIH fractions (Fig. 1*c*).

Having established that ERCC2 and ERCC3 are components of TFIIH, we investigated the possible role of ERCC2 and ERCC3 in transcription. Addition of antibodies against ERCC2 or ERCC3 to the reconstituted transcription system resulted in an inhibition of transcription (Fig. 2). This inhibition was specific because it could be overcome by addition of excess TFIIH, but not by TFIIB or TFIIE (Fig. 2). These results are consistent with the observation that the yeast homologues of ERCC2 (RAD3) and ERCC3 (RAD25) are essential for transcription[10,11].

Our finding that TFIIH contains at least two excision-repair proteins suggested that the entire TFIIH complex may partici-

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pate in excision repair. The human excinuclease removes DNA damage by hydrolysing the fifth phosphodiester bond 3′ and the 21–23rd phosphodiester bond 5′ to the lesion[12]. These incisions release a damaged DNA fragment of 27–29 nucleotides. Using an assay that measures the release of such a fragment, extracts derived from all seven XP complementation groups (XPA–XPG) (reviewed in refs 13–15) including the rodent ERCC1 mutant, were tested for excision-repair complementation using the TFIIH fraction from the last chromatographic step. Only XPB, XPC and XPD cell-free extracts were complemented by TFIIH (Fig. 3*a*). Importantly, there was an absolute coelution of TFIIH transcription activity with activities complementing XPB and XPD extracts in the last purification step (Fig. 3*b*, *c* and *e*), whereas the XPC complementing activity is shifted by three fractions (Fig. 3*d*). We conclude that XPB (ERCC3) and XPD (ERCC2) are integral subunits of TFIIH and that XPC,

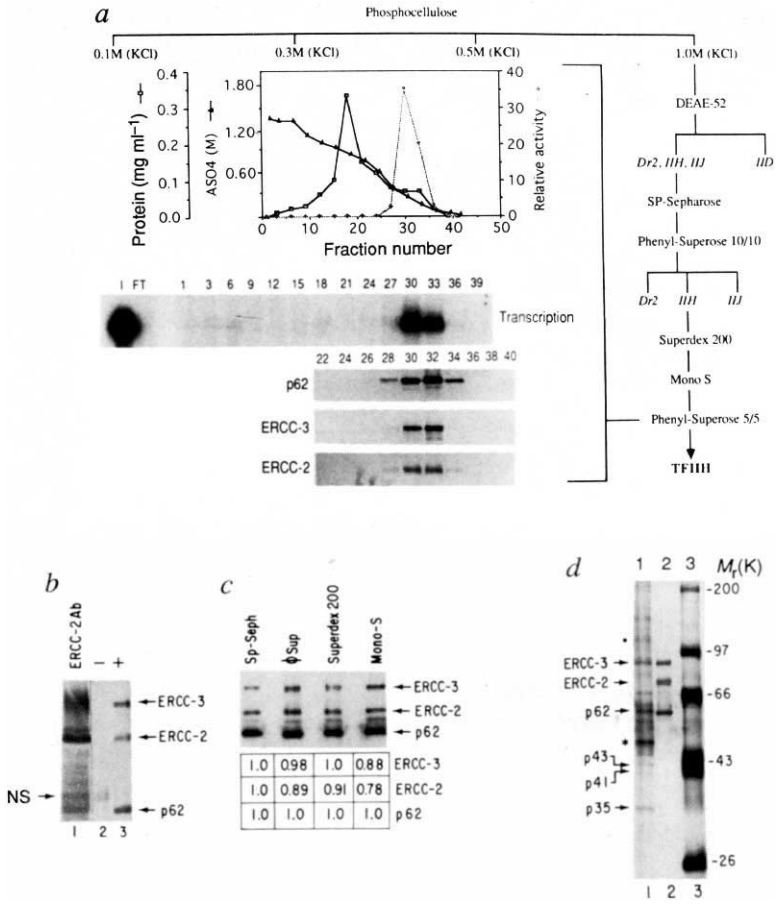

FIG. 1 *a*, TFIIH transcription activity copurifies with ERCC3, ERCC2 and p62 polypeptides. TFIIH was purified as described[26] with modifications. Transcription reactions contained the adenovirus major late promoter (Ad-MLP) directing transcription of a 392-nucleotide G-less cassette. Reactions contained all the purified general transcription factors (GTFs) and RNA polymerase II, except TFIIH. Assays were supplemented with the fraction whose number is indicated at the top of each lane. 'I', input to the column; 'FT', flow-through of the column. Co-elution of ERCC2, ERCC3 and p62 polypeptides was confirmed by western analysis. The p62 panel represents a western done with protein-A-purified p62 antibodies; ERCC2 and ERCC3 panels are westerns using affinity-purified antibodies against ERCC2 and ERCC3. *b*, Affinity-purified ERCC2 antibodies immunoprecipitate the TFIIH subunits p62 and ERCC3. NS, nonspecific band present in lane 1 and in lane 2. *c*, Quantitative western blot of p62, ERCC2 and ERCC3 polypeptides during purification. Equal amounts of p62-reactive material, as determined by quantification on a Biorad GS-250 Molecular Imager, were loaded in each lane and western blotted using all three antibodies (against p62, ERCC2, ERCC3). *d*, Composition of TFIIH. The transcriptionally active pool from the final chromatography step was subjected to SDS–PAGE followed by silver staining (lane 1). The same pool was analysed by western blotting using all three antibodies; the resulting blot is shown next to the silver-stained lane (lane 2). Size markers are shown in lane 3.

METHODS. *a*, TFIIH was purified from 10.5 g HeLa nuclear extract and fractionated on phosphocellulose as described[26,27]. *b*, 50 μl TFIIH eluted from the Superdex-200 column was incubated for 3 h at 4 °C with either protein A–agarose beads (RepliGen) alone (lane 2) or with protein A–agarose beads to which antibodies against ERCC2 had been adsorbed (lane 1). The beads were then washed 5 times with buffer containing 50 mM Tris-HCl, pH 7.6, 50 mM NaF, 100 μM $NaVO_3$, 10 mM β-glycerophosphate, 1.0% NP-40, 0.3 M NaCl, and resuspended in SDS–PAGE loading buffer. After electrophoresis proteins were transferred onto nitrocellulose and the ERCC3, ERCC2 and p62 components of TFIIH were detected with the appropriate antibodies. Lane 1 contains 10 μl of the TFIIH phenyl-Superose (last step of purification) fraction as a positive control. *c*, Quantitative western blot. Equal amounts of p62-reactive material, independently determined, were loaded in each lane: SP-Sepharose (10 μg), phenyl-Superose (8.5 μg), Superdex 200 (7.7 μg) and Mono-S (6.6 μg). Proteins were subsequently transferred onto nitrocellulose and western blotted with antibodies against p62, ERCC2 or ERCC3 using the enhanced chemical luminescence (ECL) method (Amersham). The blot was exposed to a Biorad CH screen and then imaged on a Biorad GS-250 Molecular Imager. The p62-reactive material quantified for all four column pools was equal and set at unity.

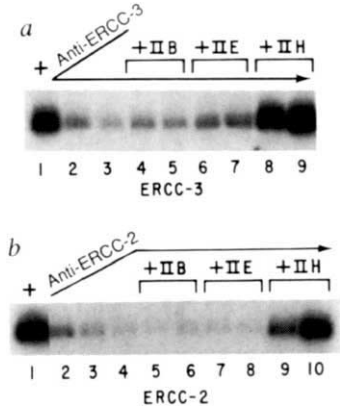

FIG. 2 Antibodies directed against ERCC2 and ERCC3 inhibit transcription. *a*, Transcription reactions were reconstituted using the general transcription factors (GTFs) and the Ad-MLP. Lane 1 is a positive control for TFIIH-dependent transcription. Lanes 2 and 3, 220 ng and 440 ng affinity-purified ERCC3 antibodies. Other lanes contain 220 ng ERCC3 antibodies in the presence of excess factors: 4 and 5, 2- and 4-fold excess TFIIB, respectively; 6 and 7, 2- and 4-fold excess TFIIE; 8 and 9, 1- and 2-fold excess purified TFIIH (1-fold is the amount of factor saturating the assay). The excess factor was not incubated with the antibodies. *b*, Lanes 2–4 contain 300, 600 and 900 ng affinity-purified ERCC2 antibodies, respectively. Other lanes contain 900 ng ERCC2 antibody in the presence of excess factors: 5–6, 2- and 4-fold excess TFIIB, respectively; 7 and 8, 2- and 4-fold excess TFIIE; 9 and 10, 1- and 2-fold excess purified TFIIH.

METHODS. Each lane contained 500 ng TFIIH from the Superdex-200 chromatography step preincubated with either ERCC2 or ERCC3 antibodies for 30 min on ice. After pre-incubation, the remaining GTFs, RNA polymerase II, DNA, nucleotide mix, and excess factors were added. Transcription reactions were incubated for an additional hour at 28 °C and processed as described[26].

although it is tightly associated with TFIIH, is not an essential subunit because it trails behind the transcription activity in the last purification step. This finding is supported by the facts that null mutants of XPB and XPD do not exist[2] and the yeast mutants of XPB (RAD25) and XPD (RAD3) are conditionally lethal[16]. In contrast, XPC null mutants have been identified in humans[17] and deletion of the XPC homologue RAD4 (ref. 18) in yeast is not lethal[16], suggesting that it is not essential for transcription. Because XPC cell-free extract is complemented by TFIIH, we tested for XPC in our TFIIH preparation. Western analysis with antibodies directed against XPC confirmed the presence of the 125K polypeptide (Fig. 1*d*, lane 1; indicated by a dot) in our TFIIH and revealed that the amount of XPC-reactive material decreased in the last step of purification as a result of its partial separation from TFIIH (data not shown).

The observation that TFIIH contains a helicase activity[1] is not surprising as XPD/ERCC2 has a 5′–3′ helicase[19] and XPB/ERCC3 has a 3′–5′ helicase (data not shown). We wished to determine the role of this activity in transcription and whether it is subject to regulation by other general transcription factors. As TFIIH contains a kinase specific for the carboxy terminal domain (CTD) of RNA polymerase II which is stimulated by TFIIE[20], we investigated the effect of this factor on TFIIH-associated helicase activity and found that it is inhibited by TFIIE (Fig. 4*a* and *b*). This inhibition seems to be selective because TFIIE can inhibit helicase activity associated with recombinant ERCC3 but not that of ERCC2 or UvrD, a factor involved in excision repair in *Escherichia coli*[21] (Fig. 4*a*, *b*). To investigate the specificity of this inhibition further, we studied the effect of antibodies directed against the two subunits of TFIIE, p34 and p56 (refs 7–9, 22). We found that preincubation of TFIIE with antibodies against p56 effectively neutralized the repressing effect on TFIIH helicase activity; antibodies against p34 had no effect (Fig. 4*c*).

Our results indicate that there could be a direct physical interaction between TFIIH (ERCC3) and TFIIE (p56). To test this possibility, TFIIE, with its 56K subunit fused to glutathione-*S*-transferase (GST), was incubated with TFIIH or with recombinant ERCC3 or ERCC2. We used a GST-pulldown assay (Fig. 4*d* legend) to demonstrate an interaction between TFIIE and ERCC3 and, as expected, with TFIIH. This interaction was specific, as recombinant ERCC2 failed to interact with TFIIE (Fig. 4*d*). Therefore, TFIIE can repress the helicase activity of TFIIH, an effect that is mediated through direct interaction of ERCC3 with the largest subunit of TFIIE. As TFIIE has no effect on ERCC2 helicase activity but inhibits that associated with TFIIH, we suggest that the ERCC2 helicase activity is dormant in the TFIIH complex.

The role of the TFIIH helicase in transcription is unclear, but our analysis suggests that TFIIH may act in open complex formation and/or promoter clearance by melting the DNA duplex at the promoter region. Our studies on the composition of the ternary complex have failed to identify XPB (ERCC3) or XPD (ERCC2) in the complex, ruling out a role for TFIIH during elongation (P. Kumar and D.R., unpublished results). It is possible that the CTD kinase and helicase activities of TFIIH function in a concerted fashion. Phosphorylation of CTD causes a conformational change in the preinitiation complex[23], which could result in TFIIH–TFIIE dissociation and unbarring of the helicase activity of ERCC3 (TFIIH); this disinhibition would enable TFIIH to catalyse open complex and/or promoter clearance.

The XPB (ERCC3) and XPD (ERCC2) proteins and their yeast counterparts RAD25 and RAD3, respectively, were

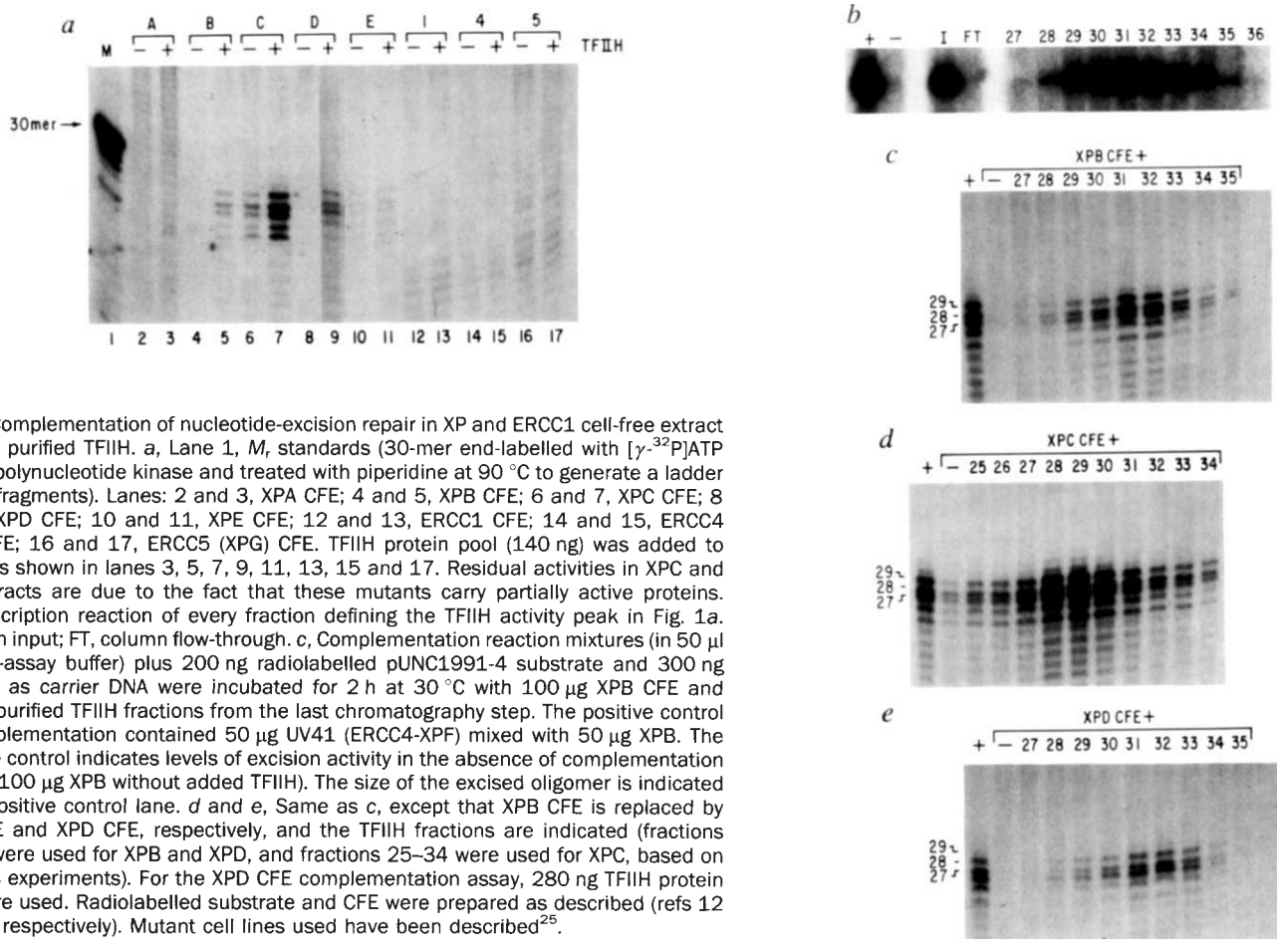

FIG. 3 Complementation of nucleotide-excision repair in XP and ERCC1 cell-free extract (CFE) by purified TFIIH. *a*, Lane 1, *M*, standards (30-mer end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase and treated with piperidine at 90 °C to generate a ladder of DNA fragments). Lanes: 2 and 3, XPA CFE; 4 and 5, XPB CFE; 6 and 7, XPC CFE; 8 and 9, XPD CFE; 10 and 11, XPE CFE; 12 and 13, ERCC1 CFE; 14 and 15, ERCC4 (XPF) CFE; 16 and 17, ERCC5 (XPG) CFE. TFIIH protein pool (140 ng) was added to reactions shown in lanes 3, 5, 7, 9, 11, 13, 15 and 17. Residual activities in XPC and XPE extracts are due to the fact that these mutants carry partially active proteins. *b*, Transcription reaction of every fraction defining the TFIIH activity peak in Fig. 1*a*. I, column input; FT, column flow-through. *c*, Complementation reaction mixtures (in 50 µl excision-assay buffer) plus 200 ng radiolabelled pUNC1991-4 substrate and 300 ng pBR322 as carrier DNA were incubated for 2 h at 30 °C with 100 µg XPB CFE and 140 ng purified TFIIH fractions from the last chromatography step. The positive control for complementation contained 50 µg UV41 (ERCC4-XPF) mixed with 50 µg XPB. The negative control indicates levels of excision activity in the absence of complementation (that is, 100 µg XPB without added TFIIH). The size of the excised oligomer is indicated in the positive control lane. *d* and *e*, Same as *c*, except that XPB CFE is replaced by XPC CFE and XPD CFE, respectively, and the TFIIH fractions are indicated (fractions 27–35 were used for XPB and XPD, and fractions 25–34 were used for XPC, based on previous experiments). For the XPD CFE complementation assay, 280 ng TFIIH protein pool were used. Radiolabelled substrate and CFE were prepared as described (refs 12 and 28, respectively). Mutant cell lines used have been described[25].

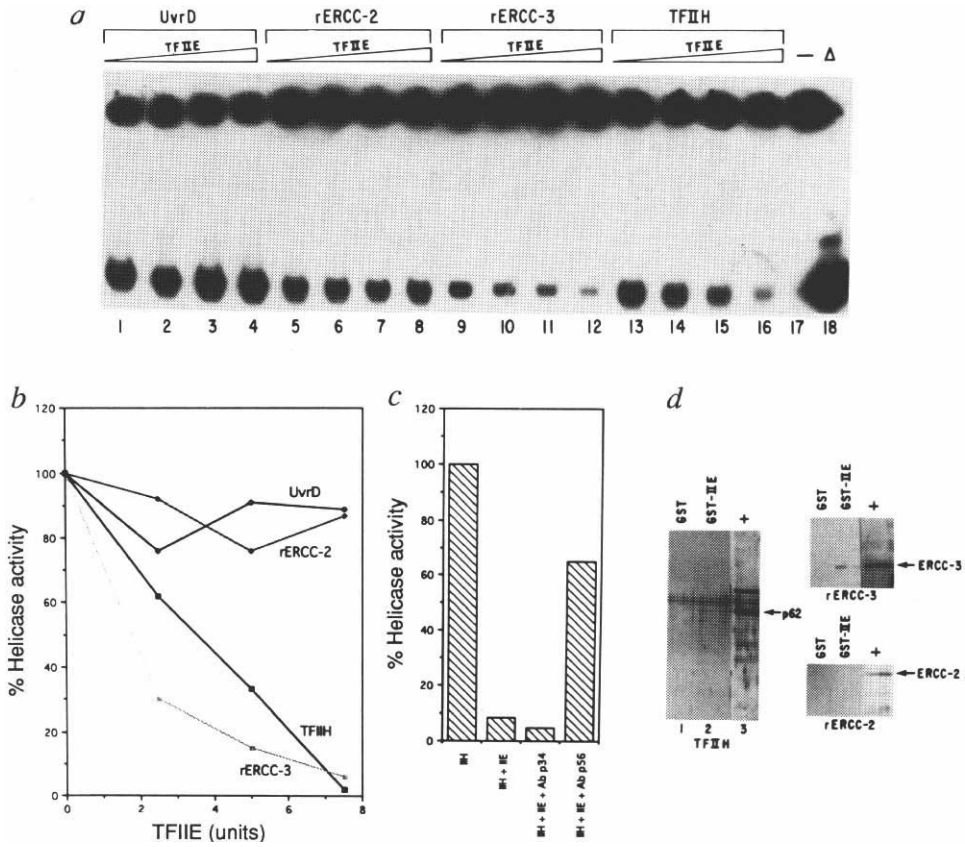

FIG. 4 TFIIH helicase activity is modulated by TFIIE. *a*, DNA helicase activity was measured by the release of a 34-nucleotide oligomer hybridized to single-stranded M13 DNA. Substrate was prepared as described[1] with modifications. *b*, Results in *a* were quantified on the Biorad GS-250 Molecular Imager and percentage helicase activity plotted against TFIIE units. *c*, Effect of antibodies directed against both subunits of TFIIE on TFIIE-dependent inhibition of TFIIH helicase activity. TFIIE was preincubated with either affinity-purified p34 antibodies or with protein A-purified p56 antibodies. *d*, TFIIH, ERCC2 and ERCC3 were tested for their ability to bind to a GST fusion protein with TFIIE-p56 (GST-56). The (+) lanes show the respective inputs of TFIIH, ERCC2 and ERCC3 to the individual experiments with TFIIE (GST-p56). GST alone serves as a negative control. The TFIIH blot was probed with p62 antibodies. The slower-migrating band in lane 3 of the TFIIH blot is a nonspecific band which is not retained by GST–p56 or by GST alone. The ERCC2 and ERCC3 blots were probed with antibodies as before.

METHODS. *a*, Helicase assay: 2 pmol single-stranded DNA were annealed to 2 nmol of a 24-nucleotide oligomer extended by Klenow fragment (15 units) in the presence of 50 μM each dGTP and dATP and 5 mCi [α-^{32}P]dCTP. After extraction with phenol and chloroform, free nucleotides were removed by gel filtration on Sephadex-G75. The DNA helicase assay (15 μl) contained 3 mM HEPES-NaOH, pH 7.9, 20 mM Tris-HCl, pH 7.9, 7 mM $MgCl_2$, 2 mM DTT, 3% glycerol, BSA at 0.5 mg ml^{-1}, 2 mM ATP and 100 ng substrate (20,000 c.p.m.) as described[1]. *b*, Results in *a* are plotted as per cent helicase activity versus TFIIE units; 0.5 μl TFIIE typically saturates our transcription assays. Therefore 0.2, 0.4, 0.6 and 0.8 TFIIE units are equal to 0.2, 0.4, 0.6 and 0.8 μl purified TFIIE. *c*, Anti-repression of helicase activity was achieved by preincubating TFIIE with either anti-p56 or anti-p34 antibodies for 45 min at 4 °C. *d*, GST pull down assay: 200-fold excess GST-p56 was incubated at 4 °C in the presence of either 10 μg TFIIH, 100 μg ERCC3 (*E. coli* with maltose-binding protein (MBP)–ERCC3 fusion), or 100 μg ERCC2 (MBP–ERCC2 fusion) induced cell-free extract for 1 h. A 50% slurry of GST resin (30 μl) was then mixed with the proteins and incubated at 4 °C for an additional 45 min with mild agitation. The GST beads were then pelleted at 3,000 r.p.m. for 2 min and washed 5 times with buffer containing 40 mM HEPES-NaOH, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.4% NP-40, 1 mM DTT and 0.5 mM PMSF. Bound proteins were separated on a 9% SDS–polyacrylamide gel and analysed by western blotting.

initially identified as nucleotide-excision repair proteins[2,16,24] before they were implicated in transcription[10,11]. The question that arises is whether the XPB and XPD proteins perform their repair function as TFIIH, as individual subunits, or as a repair complex containing subunits other than those found in TFIIH. We believe that these proteins perform their function in the form of TFIIH for the following reasons. Contrary to *in vivo* experiments in which cell fusions of XPB (ERCC3) and XPD (ERCC2) mutants yielded full complementation, cell-free extracts from XPB (ERCC3) and XPD (ERCC2) cell lines failed to complement, or gave only marginal complementation in an *in vitro* excision assay[25]. The most plausible explanation of these results is that in the *in vivo* complementation, functional TFIIH can be assembled with wild-type XPB and XPD, but *in vitro* the TFIIH complexes containing mutant XPB and mutant XPD do not readily exchange subunits, and as a result very little TFIIH with wild-type XPB and XPD exists. Indeed, our attempts to complement the XPD cell-free extract with purified XPD (ERCC2) failed, reflecting the stability of the TFIIH complex and the difficulty of exchanging subunits. We conclude that TFIIH carries out RNA polymerase II transcription and DNA repair as a functional unit. □

Received 29 December 1993; accepted 29 March 1994.

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ACKNOWLEDGEMENTS. We thank the members of our laboratories for discussion, and E. Maldonado for providing ERCC2 and ERCC3 antibodies. This work was supported by grants from the NIH (D.R., A.S. and R.D.) and by a Faculty research award from the American Cancer Society (D.R.).