All pairwise interactions of RNA polymerase II and general transcription factors (TF) IIB, E, F, and H have been quantitated by surface plasmon resonance with the use of a Ni2+ chelate on the sensor surface where necessary to attain higher sensitivity. Only 4 of 10 possible interactions were found above the detection limit: TFIIB, -E, and -F binding to RNA polymerase II and TFIIE binding to TFIIH. These four interactions constitute a minimal set for the formation of a transcription initiation complex and may represent the primary interactions involved in assembly of the complex. Point mutations were introduced in TFIIB that alter transcription start sites in vivo markedly diminished the affinity of TFIIB binding to RNA polymerase II. Protein blotting revealed an interaction between the largest subunit of TFIIE and third largest subunit of TFIIH, which may be responsible for TFIIE binding to TFIIH.

An RNA polymerase II transcription system can be reconstituted from the polymerase and five general initiation transcription factors (TF)1 IIB, D, E, F, and H (1). These proteins have been resolved to homogeneity from yeast and mammalian cells, and genes for all 28 polypeptides of the minimal system have been cloned from yeast (2–8). As the proteins assemble in a large complex at a promoter prior to the initiation of transcription, the analysis of interactions among them should be informative about the initiation mechanism. Interactions of purified transcription proteins have been investigated in the past by sedimentation, gel electrophoretic, and affinity chromatographic approaches (3, 6, 9–11). Many pairwise interactions among the polymerase and general transcription factors have been observed, but the results have remained largely qualitative and incomplete.

We have extended previous analyses to the quantitative determination of pairwise interactions among all transcription proteins except TFIIID. Our findings distinguish between strong and comparatively weak interactions and reveal a simple pattern of protein-protein contacts in the transcription initiation complex. This pattern is consistent with previous functional evidence (12) and with an ordered pathway of initiation complex assembly derived by others (1, 13–15).

EXPERIMENTAL PROCEDURES

Construction of Hexahistidine-tagged RPB1 Gene and Purification of Tagged RNA Polymerase II from S. pombe Strain—The RPB1 gene was amplified by a polymerase chain reaction with the primers 5′-TCG CAT GAT ATA GTA TAT CTT T3′, 5′-CCC CTC GTA CCG GTG CAT TTG ATG TG-3′, 5′-AGC TTCG CAT CTC GAG TGG TGA TGG TGA TGG TGA TGG GAA TTT TCA TTT TCA T3′, and 5′-CCC CAA GCT TAG AAG TTA CCG GGA GA-3′, introducing an Spel restriction site and adding six histidines at the C terminus. The KpnI-HindIII region of RPB1 in pSP1 (16) was replaced by the two polymerase chain reaction products, the XbaI-HindIII fragment of the resulting plasmid was inserted between the XbaI and HindIII sites of YCP57, and the resulting plasmid was transformed into ZB6 (MAT a ura3-52 leu2-3 leu2-112 his3-300 rpb1-187: HIS3 prp112: URA3 CEN 4 RPB1) as described (16). RPB4 was then knocked out in this strain and in CB010 (MAT a pep4::HIS3 prb1::LEU2 prc::HIS3 can1 ade2 trp1 ura3 his3 leu2-3,112 ci GAL 1 RAS 1 SUC 1) with the BamHI fragment of pSpL9 (17). Southern blot analysis of genomic DNA confirmed that homologous recombination had occurred.

RNA polymerase II was purified as described previously with the addition of 10 μM ZnCl2 to all buffers (18). C-terminal domain (CTD)-less RNA polymerase II was generated as described (16).

Co-expression of TFIIIE Subunits and Purification of Recombinant Protein—TFA2 (4) was amplified by a polymerase chain reaction with the primers 5′-ATA TCC ATG GCT AGT AAA AAC AGG GAC CCT-3′ and 5′-ATA TGG ATC CTC ATA TCT GAG ATT AA-3′, introducing NcoI and BamHI restriction sites at the 5′- and 3′-ends of the open reading frame. The resulting fragment was cloned in pET-11d (Novagen). A double expression plasmid for both subunits of TFIIIE was constructed by cloning the Spel/blunted-PvuI fragment of the TFA2 pET-11d plasmid into the Spel-PvuI site of a hexahistidine-tagged TFA1 pET-11a plasmid (4).

B. d. e.3 cells were transformed with the hexahistidine-tagged TFA1 TFA2 plasmid, grown in culture to an A600 of 0.5 at 37 °C, induced with 0.4 μM isoprpyl-β-D-thiogalactopyranoside at 30 °C for 3 h, harvested, resuspended in 40 ml of lysis buffer (20% glycerol, 20 μM Tris-Cl, pH 8.0, 100 mM NaCl, 0.1% Tween 20, 2 mM imidazole, 5 mM β-mercaptoethanol), and lysed by sonication. The lysate was clarified by centrifugation and loaded onto a Poros MCM 10 × 100 min column (PerSeptive Biosystems) charged with Ni2+. The column was washed with 16 ml of lysis buffer, 16 ml of wash buffer (20% glycerol, 100 mM sodium phosphate, pH 6.3, 500 mM NaCl, 0.01% Nonidet P-40, 2 mM imidazole) containing 500 mM NaCl, 16 ml of wash buffer containing 100 mM NaCl, and 16 ml of elution buffer (20% glycerol, 20 μM Tris-Cl, pH 7.6, 100 mM NaCl, 0.01% Nonidet P-40) containing 10 mM imidazole. TFIIIE was eluted with a 5-column volume gradient of elution buffer containing 10–100 mM imidazole. Peak fractions were pooled; diluted 2-fold with 10 mM potassium phosphate, pH 6.3, 20% glycerol, 100 mM potassium acetate; and loaded on a 5-ml hydroxyapatite column (Bio-Rad), developed as described (19).

For analysis by gel filtration, 10 μl of 0.1 mg/ml TFIIIE was injected onto a Bio-Sil SEC 400 300 × 7.5 mm column (Bio-Rad) at 0.2 ml/min in running buffer (40 mM Hepes, pH 7.6, 7.5 mM MgCl2, 120 mM potassium acetate, and 0.005% Surfactant P-20). The apparent molecular mass was 105 kDa on the basis of comparison with gel filtration standards (Bio-Rad).

TFIIIB, TFIIF, and TFIIH—Recombinant TFIIIB was prepared as

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** The abbreviations used are: TF, transcription factor(s); CTD, C-terminal domain; TBP, TATA-binding protein.
described (3). Mutant forms of TFIIB, Sua7-1, and Sua7-3 (21) were prepared as described. TFIIF was isolated from yeast (6). Core TFIIH was prepared by the first of two procedures as described (22).

Surface Plasmon Resonance—Surface plasmon resonance measurements of protein-protein interactions were performed with the BIAcore Biosensor (Pharmacia Biotech Inc.). All measurements were at room temperature in running buffer (40 mM Hepes, pH 7.6, 7.5 mM MgCl₂, 120 mM potassium acetate, 0.005% Surfactant P-20) with a flow rate of 15 μl/min. Proteins were immobilized on CM5 research grade sensor chips with the use of an amine coupling kit (Pharmacia) (23, 24).

Polymerase was immobilized in 100 mM sodium acetate, pH 5.0; TFIIE was immobilized in 100 mM sodium acetate, pH 5.0; TFIIB was immobilized in 100 mM sodium acetate, pH 6.0; and TATA-binding protein (TBP) was immobilized in running buffer. Blank surfaces were prepared by the introduction of buffer without protein. Noncovalently bound proteins were removed by washing with running buffer containing 1 M potassium acetate. All proteins were diluted in running buffer from concentrated stock solutions prior to use.

A Ni²⁺ chelate sensor chip was prepared as described (25). For immobilization of RNA polymerase II and TFIIIE, the hexahistidine-tagged proteins (50 μg/ml) were passed over the surface at 1 μl/min in running buffer containing 2 mM imidazole. All binding measurements were performed at 15 μl/min in the same buffer. Data were analyzed with BIAevaluation, Version 2.1, using the AB₁B and A₁B₅ models for dissociation and association (Pharmacia).

RESULTS

Protein-protein interactions were quantitated by surface plasmon resonance, which detects the change in refractive index near a surface bearing one adsorbed protein due to interaction with another protein. The surface is typically coated with a dextran layer, adsorbing the first protein nonspecifically and therefore in a variety of orientations. The refractive index change on binding of the second protein depends on its size and proximity to the surface; and for a single site, as in the cases studied here, the change is proportional to the amount of bound protein. The refractive index change, expressed in resonance units, may be recorded as a function of time after the addition of a solution of the second protein or following the removal of this solution for determination of rates of association and dissociation, respectively. These rates are related through simple exponentials to the fundamental rate constants $k_a$ and $k_d$ for the binding reaction,

$$k_d = \frac{A + B}{AB} = \frac{k_d}{k_a}$$ (Eq. 1)

with $k_d/k_a = K_a$, the equilibrium association constant. A test of validity of the analysis is the linearity of $\ln(R/R_0)$ versus time for the dissociation reaction, where $R$ is the detector response and $R_0$ is its value at the start of dissociation. In most cases studied here, linearity was observed, except for deviations due to bulk refractive index changes during the first 30 s of association reactions and more persistent deviation for RNA polymerase II-TFIIE interaction discussed below (Fig. 1).

RNA Polymerase II Transcription Protein Interactions

| Rate constants for dissociation ($k_d$) and association ($k_a$) of RNA polymerase II-general transcription factor complexes. Dissociation and association rate constants ($k_d$, $k_a$) for general transcription factors TFIIE, TFIIB, TFIIF, and TFIIH (in the range of concentrations indicated) interacting with immobilized RNA polymerase II (27, 18, 15, and 2 determinations, respectively) are shown. std. dev., standard deviation; n.d., not detected. |
|---|---|---|---|---|
| E | $3.5\times10^{-3}$ | 9.1×10⁻⁴ | 1.4×10⁵ | 7.3×10⁴ | 0.05-5.5 μM |
| B | $4.5\times10^{-3}$ | 2.9×10⁻⁴ | 1.8×10⁵ | 1.4×10⁵ | 0.5-2.5 μM |
| F | $3.8\times10^{-4}$ | 8.5×10⁻⁵ | 6.4×10⁵ | 1.7×10⁵ | 3-30 nM |

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2 Leuther, K., Bushnell, D., and Kornberg, R. (1996) Cell 85, 773-779.
RNA polymerase II-TFIIE interaction was readily detectable by surface plasmon resonance effect (Fig. 1), with a variation in slope corresponding to a 3-fold range of $k_d$ values. This variation could not be accounted for by the presence of multiple TFIIIE-binding sites but might reflect conformational mobility, heterogeneity, or some other aspect of the proteins under study (see "Discussion"). In view of the dependence of human TFII E binding on the phosphorylation state of the polymerase CTD, we tested yeast TFII E binding to polymerase lacking a CTD, as well as to polymerase lacking subunits 4 and 7, and obtained apparent $K_a$ values of $1.4 \times 10^7$ and $7.4 \times 10^6$ M$^{-1}$, respectively, which differs little from $K_a$ for the native polymerase (Fig. 4). TFII E binding to CTD-less polymerase and A 4/7 polymerase showed the same variance in dissociation as TFII E binding to normal RNA polymerase II (data not shown). We did note an influence of the nature of the salt upon RNA polymerase II-TFII E interaction, with potassium acetate most favorable, followed by ammonium sulfate and sodium chloride (Fig. 5). It may be significant that the same order of preference for these salts has been found in transcription.

For the studies of TFII E interaction reported here, recombinant protein was isolated from a bacterial strain co-expressing the large (Tfa1) and small (Tfa2) subunits. A mass of 105 kDa was estimated by gel filtration. In order to determine the affinities of the individual subunits for RNA polymerase II, they were separately expressed and purified. Tfa1 bound native, CTD-less, and subunits 4 and 7-deficient polymerases with $K_a$ values of $7.8 \times 10^6$, $1.1 \times 10^7$, and $1.1 \times 10^7$ M$^{-1}$, respectively, which are comparable to the affinities measured for intact TFII E. Tfa2 exhibited no appreciable binding at all.

RNA polymerase II-TFII E interaction, well documented in many previous studies (6, 10, 11), could not be assessed with polymerase bound to a dextran surface because the concentrations of pure yeast TFII F available and the $k_d$ were low, and the surface plasmon resonance effect was consequently near the lower limit of detection. We turned to the use of a sensor chip derivatized with a Ni$^{2+}$ chelate for binding hexahistidine-tagged proteins (23). Omission of the dextran layer brings bound proteins closer to the chip surface, enhancing the reso-
nance response. RNA polymerase II with a hexahistidine tag at the end of the CTD was engineered and bound to the Ni\(^{2+}\) chip. TFIIF binding could then be detected (Figs. 1, 2, and 4) with a sensitivity approximately 20-fold greater than that with the use of the dextran chip.

RNA polymerase II-TFIIH interaction was investigated with preparations of core TFIIH, a 5-subunit protein that included a trace of a sixth subunit, Ssl2 (22). No evidence of interaction was detected. Taking into account the concentration of TFIIH used, \(k_a\) for RNA polymerase II-TFIIH must be less than about \(10^4 \text{M}^{-1}\text{s}^{-1}\), corresponding to \(K_a\) less than or on the order of \(10^6\) M\(^{-1}\).

General Transcription Factor-Factor Interactions—The only interaction among the general transcription factors detectable above the approximate limit was between TFIIE and TFIIH (Figs. 1 and 4). The binding of TFIIH (concentration range, 5-100 nm) to immobilized TFIIIE occurred with a \(k_a\) value of \(3.3 \times 10^3 \text{s}^{-1}\text{M}^{-1}\) (standard deviation was \(1.2 \times 10^3\)) and a \(k_d\) value of \(1.1 \times 10^{-3} \text{s}^{-1}\) (standard deviation was \(1.9 \times 10^{-4}\)) (results of 15 determinations). To identify the subunits of these proteins involved in the interaction, Tfa1 and Tfa2 were individually immobilized on dextran chips. Tfa1 bound TFIIH with nearly the same affinity as intact TFIIIE, whereas Tfa2 failed to bind TFIIH at all. Tfa1 interacted specifically with the Tfb1 subunit of TFIIH in a protein (Western) blot (Fig. 6).

DISCUSSION

The analysis by surface plasmon resonance reported here has revealed 4 of 10 possible pairwise interactions among RNA polymerase II transcription proteins (Fig. 4): TFIIB, -E, and -F bind RNA polymerase II, and TFIIIE binds TFIIH. No other interaction was detectable above an approximate limit for an equilibrium association constant of \(10^6\) M\(^{-1}\). The four interactions observed constitute a minimal set for formation of a complete transcription initiation complex since these interactions connect all five proteins studied here, and the remaining component of the complex, TFIID, is incorporated through contacts with both TFIIIB and promoter DNA. The initiation complex formation guided by four primary interactions is consistent with the assembly pathway defined by others (1, 13–15). TFIID, and in particular its TBP component, binds to the TATA box in promoter DNA that creates a context for TFIIIB binding. TFIIIB then binds RNA polymerase, as do TFIIIE and TFIIF, followed by TFIIH binding to TFIIE. Additional interactions, while dispensable for complex formation, are not ruled out. They may occur in the context of a ternary or higher complex either as a consequence of conformation changes or because they are of much lower affinity, below the limit of detection in this study, but persist when they are bridged by the primary interactions reported here.

The four primary interactions are all likely to be specific in view of the effects of mutations in TFIIIB reported here and the results of functional and structural studies presented elsewhere. TFIIB-RNA polymerase II and TFIIE-TFIIH interactions in yeast are required for the initiation of transcription in vitro (12). TFIIE-TFIIH interaction has also been reported to stimulate TFIIH kinase activity, and TFIIF-RNA polymerase II interaction influences template binding (12, 20, 28–30). Electron crystal structures of TFIIB- and TFIIIE-RNA polymerase II complexes show single sites of transcription factor binding to the polymerase.2

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REFERENCES
1. Conaway, R. C., and Conaway, J. W. (1993) Annu. Rev. Biochem. 62, 161–190
2. Gileadi, O., Feaver, W. J., and Kornberg, R. D. (1992) Science 257, 1389–1392
3. Tschochner, H., Sayre, M. H., Flanagan, P. M., Feaver, W. J., and Kornberg, R....
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