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Simple derivation of TFIID-dependent RNA polymerase II transcription systems from *Schizosaccharomyces pombe* and other organisms, and factors required for transcriptional activation

(*Saccharomyces cerevisiae*/human T cells/*in vitro* transcription system/acidic activators/transcription factor TFIID)

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ABSTRACT **Resolution of whole cell extract through two chromatographic steps yields a single protein fraction requiring only the addition of TFIID for the initiation of transcription at RNA polymerase II promoters. This approach allows the convenient generation of RNA polymerase II transcription systems from *Saccharomyces cerevisiae*, human lymphocytes, and *Schizosaccharomyces pombe*. TFIIDs from all three organisms are interchangeable among all three systems. The *S. cerevisiae* and *Sch. pombe* systems support effects of acidic activator proteins, provided a further protein fraction from *S. cerevisiae* is supplied. This further fraction is distinct from the mediator of transcriptional activation described previously and represents a second component in addition to general initiation factors that may facilitate a response to acidic activators.**

Transcription by RNA polymerase II in the fission yeast *Schizosaccharomyces pombe* bears a greater resemblance to that in higher cells than to the process in the budding yeast *Saccharomyces cerevisiae*. Initiation by RNA polymerase II in both *Sch. pombe* and higher cells usually occurs at a unique site 25–40 bp downstream of a single TATA element (1). When mammalian promoters are introduced into *Sch. pombe*, initiation occurs at the same sites as those utilized in mammalian cells (2, 3). By contrast, initiation in *S. cerevisiae* occurs 40–120 bp downstream of the TATA element (4); there are frequently multiple initiation sites and often multiple TATA elements as well. *Sch. pombe* is therefore particularly attractive as an organism amenable to genetic and biochemical analysis for elucidating mechanisms of RNA polymerase II transcription in mammalian cells.

Recent analyses of RNA polymerase II transcription in *S. cerevisiae* further motivate study of the process in *Sch. pombe*. Activities have been identified in extracts from *S. cerevisiae* that substitute for general initiation factors TFIIA (5) and TFIID (6, 7) in a mammalian (HeLa cell) transcription system. Genes encoding yeast and human TFIIDs were cloned on this basis (8–14), and the products of these genes are functionally interchangeable between *S. cerevisiae* and HeLa systems *in vitro* (6, 15, 16). It has not proved possible, to date, to exchange other initiation factors between *S. cerevisiae* and mammalian systems, and therefore *Sch. pombe*, which so closely resembles mammalian cells in regard to polymerase II transcription *in vivo*, becomes of particular interest for studies of transcription *in vitro*. The chief obstacle to such studies has been a lack of success in preparing extracts from *Sch. pombe* that support initiation of transcription at polymerase II promoters. We report here how this obstacle may be overcome, and exploit the *Sch. pombe* transcription system to demonstrate the dependence of activated transcription upon a factor that is functionally conserved across species.

MATERIALS AND METHODS

Yeast Strains and Plasmids. *S. cerevisiae* strain BJ926 was used (17), and *Sch. pombe* strain YNN36 (*leu1-32, ura4-294, ade6-216*) was provided by Marj Thomas and Ronald W. Davis (Stanford University). Templates for reactions with the *Sch. pombe* fraction contained an oligonucleotide sequence from the *Sch. pombe ADH* promoter region (corresponding to nucleotides −130 to −60; see figure 2 of ref. 1) except that the G residue at −65 was changed to an A and the G residue at −61 to a C. This synthetic promoter was blunt-end ligated to the G− cassette sequence (18) and cloned in pSP73 (19). A GAL4-binding site (20) or a tandem repeat of a GCN4 consensus site (19) was cloned upstream of the synthetic promoter/G− construct to give rise to plasmids pGAL4ADHG− and p(GCN4)$_2$ADHG−. Plasmid pMLG−, containing a minimal adenoviral major late promoter sequence, was constructed as described (21).

Extracts and Chromatographic Fractions. *S. cerevisiae* and *Sch. pombe* strains were grown in 12 liters of YPD (2% peptone/1% yeast extract/2% glucose) at 30°C for seven to eight generations to an OD_{600} of 8 (*S. cerevisiae*) or of 3 (*Sch. pombe*). Cells were harvested, washed once with 1 liter of cold distilled water, and suspended in 3× lysis buffer (1 ml per 3 g of cells) with protease inhibitors and with 0.5 M potassium acetate in place of 0.4 M KCl (22). About 240 ml of cell suspension was placed in a 400-ml stainless steel bead beating chamber (Biospec) with sufficient glass beads to fill the chamber. Cells were disrupted with 20 cycles of bead beating for 30 sec and cooling for 90 sec. The temperature of the surrounding ice/salt bath was kept at −10°C. Glass beads and cellular debris were removed by centrifugation in a Beckman JA10 rotor at 8000 rpm for 15 min at 4°C, and the supernatant was clarified by centrifugation in a Beckman Ti45 rotor at 42,000 rpm for 90 min at 4°C. (During decanting of the supernatant, care was taken not to include a layer of loose sediment near the pellet at the bottom of the Ti45 tube.) The protein concentration of the resulting high-speed supernatant (whole cell extract, WCE) was about 30 mg/ml.

WCE (2 g of protein) was diluted 4-fold with buffer A containing protease inhibitors (15) and loaded on a Bio-Rex 70 (Bio-Rad) column (≈40 mg of protein per ml of resin) at 2 column volumes per hr. The Bio-Rex and subsequent DE52 (Whatman) columns were washed and eluted with buffer A containing the potassium acetate concentration indicated in parentheses (3 column volumes in each case). The Bio-Rex

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Abbreviations: WCE, whole cell extract; yTFIID, *S. cerevisiae* TFIID; pTFIID, *Sch. pombe* TFIID; hTFIID, human TFIID.

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buffer A(600) eluate (120 mg of protein) was dialyzed against buffer A containing 20 mM Tris acetate (pH 7.9) in place of Hepes (pH 7.6), to a conductivity equivalent to buffer A containing 100 mM potassium acetate, and was loaded on a DE52 column (20 mg of protein per ml of resin). The column was washed with buffer A(200) and eluted with buffer A(350). The peak protein fractions were pooled and used without further manipulation in transcription reactions. This Bio-Rex–DE52 fraction typically contained about 3 mg of protein per ml. Fraction c was purified from nuclear extract as described (15). Previously, we had found that the flowthrough fraction from WCE chromatographed over Bio-Rex 70 was inhibitory to transcription, and so fraction c activity has not been derived from WCE.

Jurkat cells were cultured as described (23) and WCE was prepared as follows. Cells were harvested in a clinical centrifuge at 1500 rpm for 10 min at 4°C, suspended in 50 ml of phosphate-buffered saline containing 0.5 mM $MgCl_2$, and centrifuged again at 3500 rpm for 10 min. The pellet (2.5 ml) was suspended in 4 volumes of 10 mM Tris chloride/1 mM EDTA, pH 8.0, containing 1 mM dithiothreitol and protease inhibitors and was incubated on ice for 20 min. The cell suspension was homogenized manually with eight strokes of a 15-ml homogenizer with a B pestle. After homogenization, 4 volumes of Manley buffer (23) and 1 volume of saturated ammonium sulfate (about 3.9 M) were added, and the homogenate was stirred for 10 min at 4°C and then centrifuged in a Beckman Ti60 rotor at 50,000 rpm for 3 hr at 4°C. Protein was precipitated from the supernatant by the addition of solid ammonium sulfate (0.33 g/ml), and the precipitate was collected by centrifugation in a Beckman SW28 rotor at 25,000 rpm for 30 min at 4°C. The pellet was resuspended in buffer A (15) and dialyzed against buffer A until the conductivity was equivalent to buffer A containing 100 mM potassium acetate. The entire preparation (150 mg of protein) was chromatographed over Bio-Rex 70 and DE52 resins as described above.

TFIID. *S. cerevisiae* TFIID (yTFIID) was purified to homogeneity from a T7 polymerase overexpression vector as described by Kelleher *et al.* (16). The *Sch. pombe* and human TFIID (pTFIID and hTFIID) genes were expressed from the T7 polymerase vector pET11a (13, 24). Cells for both preparations were broken by sonication in buffer C [50 mM Hepes, pH 7.6/10% (vol/vol) glycerol/1 mM EDTA/0.5 mM dithiothreitol/1 mM phenylmethanesulfonyl fluoride] containing 300 mM KCl, followed by passage over DE52 in high salt (buffer C with 300 mM KCl) to remove nucleic acids. The extracts were dialyzed to a conductivity equivalent to 100 mM KCl and loaded on a heparin-Sepharose column equilibrated in the same buffer. The column was washed with buffer C containing 300 mM KCl and the TFIID proteins were eluted with buffer C containing 600 mM KCl. As judged by SDS/PAGE and staining with Coomassie blue, pTFIID was at least 80% pure and hTFIID was about 25% pure.

RESULTS

A Protein Fraction from WCE Requiring Only TFIID for Transcription. In the course of fractionating an RNA polymerase II transcription system from *S. cerevisiae* (15), we found that several general initiation factors copurified through two steps. A single protein fraction was derived from WCE by ion-exchange chromatography on Bio-Rex 70 and DE52 resins (Fig. 1) that required only the addition of TFIID for promoter-dependent RNA polymerase II activity (ref. 16 and Fig. 2). The several general initiation factors in the active Bio-Rex–DE52 fraction were enriched ≈200-fold from the starting extract. Copurification to this extent may reflect an association of the factors in a larger complex, but these can be resolved on further chromatography (M.H.S., unpublished observations).

Remarkably, the same procedure yielded active protein fractions from extracts of *Sch. pombe* and of a human lymphocyte (Jurkat) cell line (Fig. 2). Previously it had been shown that nuclear extracts made from Jurkat cells accurately initiate transcription (26). Transcription reactions were supplemented with bacterially expressed TFIID from the same organism as the Bio-Rex–DE52 fraction. The level of transcription was similar for *S. cerevisiae, Sch. pombe*, and Jurkat fractions. Transcription was judged to be authentic for the following reasons. First, transcription was abolished by α-amanitin at 10 μg/ml for the *S. cerevisiae* and *Sch. pombe* fractions and at 1 μg/ml for the Jurkat fraction, showing that the reaction was due to RNA polymerase II (Fig. 2). Second, transcription was absolutely dependent upon the addition of TFIID (Fig. 2). Third, transcription required a promoter, which could be either the adenoviral major late promoter (Fig. 2) or the *Sch. pombe ADH* promoter (data not shown), in the case of the *Sch. pombe* fraction. Finally, primer extension mapping confirmed that initiation occurred with the *Sch. pombe* and Jurkat fractions at the same site in the adenoviral major late promoter as previously reported for the HeLa transcription system (data not shown).

The derivation of an active Bio-Rex–DE52 fraction from Jurkat cells was surprising in light of the chromatographic behavior reported for TFIIB from HeLa cells. TFIIB and TFIIE are eluted at the same ionic strength from phosphocellulose. The two activities are then resolved on DEAE-

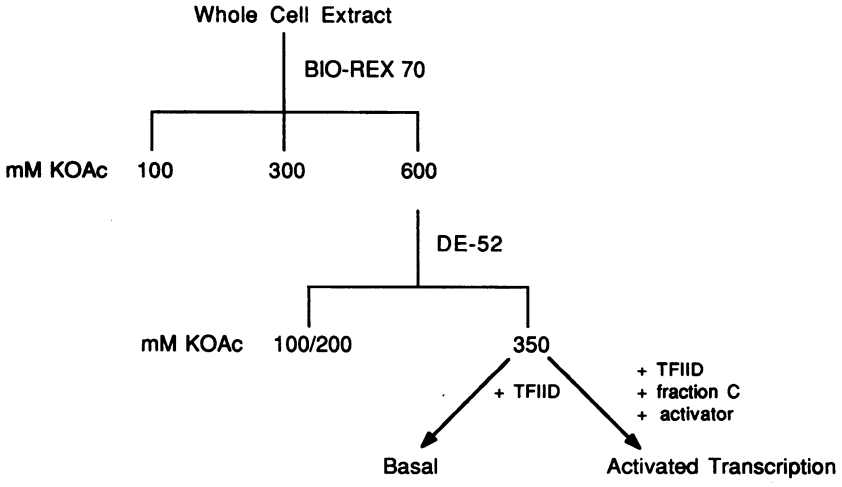

FIG. 1. Scheme for deriving an RNA polymerase II transcription system from WCE.

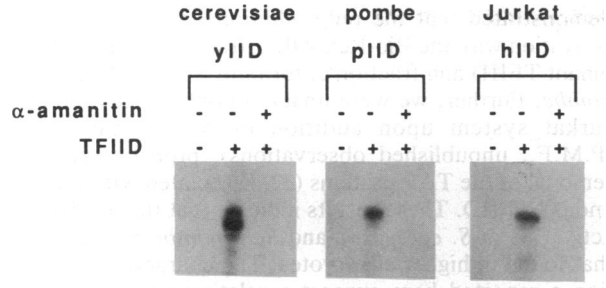

FIG. 2. Basal transcription with Bio-Rex–DE52 fractions from *S. cerevisiae*, *Sch. pombe*, and Jurkat cells. For fractions from *S. cerevisiae* the template was 0.3 μg of pΔCG− (21), and for *Sch. pombe* and Jurkat fractions 0.3 μg of pMLG− template (21) was used. Reaction mixtures (35 μl) contained 8–10 μg of Bio-Rex–DE52 fraction and 75 ng of pTFIID, yTFIID, or hTFIID and were incubated and processed as described (21, 25).

Sephacel, with TFIIB flowing through and TFIIE eluted in 300 mM KCl (27). We found no requirement for the DE52 flowthrough fraction in transcription with our preparations from Jurkat cells. Perhaps the Bio-Rex–DE52 procedure is less disruptive of a larger complex of general initiation factors than phosphocellulose and DEAE-Sephacel.

***S. cerevisiae*, *S. pombe*, and Human TFIIDs Are Functionally Interchangeable for Basal and Activated Transcription.** As mentioned above, *S. cerevisiae* and human TFIIDs are functionally interchangeable between *S. cerevisiae* and HeLa transcription systems. This functional conservation reflects a high degree of sequence conservation—>80% identity of 180 C-terminal amino acids among six cloned TFIIDs, including *S. cerevisiae*, *Sch. pombe*, and human TFIIDs (8–14, 24, 28–30). We therefore expected and indeed found that *S. cerevisiae* and human TFIIDs could be substituted in the *Sch. pombe* system (Fig. 3 *Left*). Further, we found that *S. cerevisiae* and *Sch. pombe* TFIIDs would support activity of the Bio-Rex–DE52 fraction from Jurkat cells and that *Sch. pombe* and human TFIIDs were effective with the fraction from *S. cerevisiae* (data not shown).

The Bio-Rex–DE52 fraction from *S. cerevisiae* was not only active in basal level transcription from a minimal promoter but would support effects of activator proteins bound to upstream sequences as well. For example, GAL4-VP16 stimulated transcription 48-fold (Fig. 4), comparable to or greater than the degree of stimulation obtained with nuclear extracts. The Bio-Rex–DE52 fraction from *Sch. pombe* also supported effects of acidic activators, both GAL4-VP16 and GCN4 (19) (Fig. 4, 30- and 35-fold stimulation of transcription, respectively). Elsewhere we have reported that yTFIID and hTFIID are functionally interchangeable in support of activation in the *S. cerevisiae* system (16). Here we show that

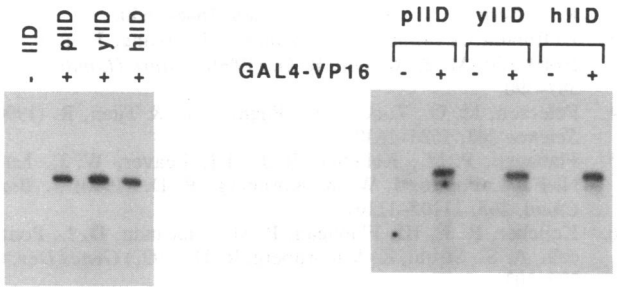

FIG. 3. yTFIID and hTFIID substitute for pTFIID in support of transcription with Bio-Rex–DE52 fraction from *Sch. pombe*. (*Left*) Reaction conditions were as described in the legend to Fig. 2; 0.3 μg of pMLG− was used as template. (*Right*) Reaction conditions for activation are as described in the legend to Fig. 4, with 0.1 μg of pGAL4ADHG−.

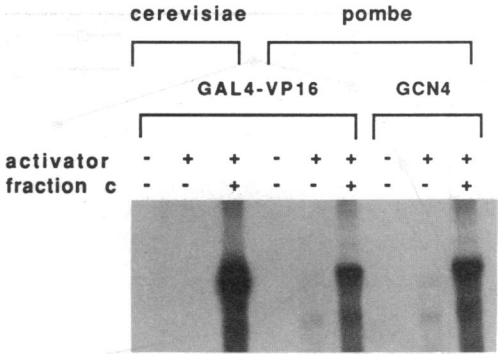

FIG. 4. Effects of acidic activators on transcription with Bio-Rex–DE52 fractions from *S. cerevisiae* and *Sch. pombe* dependent on the addition of *S. cerevisiae* fraction c. The template for *S. cerevisiae* reactions was 0.1 μg of pGAL4CG− (21, 25). Templates (0.1 μg) pGAL4ADHG− and p$(GCN4)_2$ADHG− were used for the *Sch. pombe* reactions. In assays containing activator, 1.2 pmol of GAL4-VP16 and 0.6 pmol of GCN4 were used. Seventy-five nanograms of yTFIID and pTFIID and 3 μg of fraction c were added.

recombinant yTFIID and hTFIID support activation by GAL4-VP16 with the *Sch. pombe* Bio-Rex–DE52 fraction (Fig. 3 *Right*). The levels of activation obtained with pTFIID, yTFIID, and hTFIID were 20-fold, 14-fold, and 12-fold, respectively. In view of the evolutionary divergence of the N-terminal region of TFIID among these organisms, these results contradict the suggestion that the N-terminal region of TFIID is required for transcriptional activation (14). Further evidence that the N-terminal region of TFIID is dispensable for basal and activated transcription has come from the functional sufficiency of N-terminally deleted TFIID *in vitro* and *in vivo* (16, 31–35).

Factors Required for Transcriptional Activation. The Bio-Rex–DE52 fraction contains mediator protein, since addition of this component, unnecessary for basal transcription but essential for activation (19, 25), was not required for the response to acidic activators (Fig. 4). It did, however, prove important to add one further component, fraction c derived as previously described (15) from *S. cerevisiae* nuclear extract (Fig. 4). Fraction c differs from mediator in that it is chromatographically distinguishable and further in that it fails to relieve activator interference. Fraction c therefore may represent, along with mediator, a second protein fraction that influences the response to acidic activator proteins in the *S. cerevisiae* system. Activation in the *Sch. pombe* system required the addition of fraction c, which was derived from *S. cerevisiae*, demonstrating the functional conservation of this component across species.

Low levels of fraction c had a small stimulatory effect on basal transcription in the *S. cerevisiae* system, whereas higher levels were slightly inhibitory (Fig. 5). Fraction c may, like the USA fraction from HeLa cells (36), contain both positive and negative regulatory factors. Fraction c further resembles USA in regard to possible interaction with TFIID: some activation could be observed in the absence of fraction c at very low TFIID concentrations (one-fifth that used in the experiments reported here). A final assessment of the importance of fraction c for activated transcription awaits reconstitution of the reaction with homogeneous components.

DISCUSSION

The several Bio-Rex–DE52 fractions described here provide a convenient basis for assay of both TFIID and fraction c. The Bio-Rex–DE52 fraction from *Sch. pombe* may itself be further fractionated to resolve general initiation factors

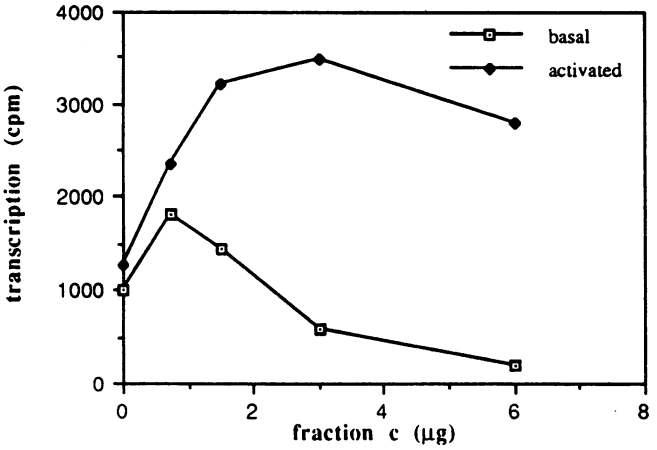

FIG. 5. Effect of fraction c on basal and activated transcription with the Bio-Rex–DE52 fraction from *S. cerevisiae*. The indicated amounts of fraction c were added to *S. cerevisiae* Bio-Rex–DE52 fraction, along with yTFIID (composed as in Fig. 2) in the absence or presence of GAL4-VP16 (5 pmol). Reaction mixtures contained 30 ng of pGAL4CG−. Radioactivity (cpm) incorporated into specific transcripts is plotted as a function of fraction c concentration.

(H.T., unpublished data). As mentioned above, the availability of these factors may contribute significantly to the elucidation of RNA polymerase II transcription in higher organisms. Finally, the applicability of the Bio-Rex–DE52 procedure to *S. cerevisiae*, *Sch. pombe*, and Jurkat cells suggests the method may be general and may thus facilitate the analysis of RNA polymerase II transcription in other plant and animal systems.

The derivation of a chromatographic fraction containing RNA polymerase II and all the general initiation factors except TFIID may have significance beyond the use in transcription reported here. Copurification of polymerase and general factors through Bio-Rex and DE52 steps, which remove 99% of the starting protein, might reflect the association of all these components in a large complex. Two further observations strengthen the notion of such a complex. First, as mentioned above, our active fraction was obtained by adsorption and elution from DE52, whereas human TFIIB flows through DE52. We have recently found (H.T., M.H.S., P.M.F., W. J. Feaver, and R.D.K., unpublished work) that the yeast homolog of human TFIIB adsorbs to DE52 in a crude state but flows through when highly purified. Moreover, pure yeast TFIIB binds specifically to RNA polymerase II, which does adsorb to DE52, and so a larger complex containing both molecules may exist initially but be disrupted in the course of fractionation. Second, at least two of the general initiation factors cosediment as an ≈400-kDa species in glycerol gradient analysis of the Bio-Rex–DE52 fraction (R.J.K., unpublished observations), although both factors sediment more slowly following extensive purification. The existence of a preformed assembly of polymerase and initiation factors would have important implications for the mechanism of initiation and its regulation.

Evidence for intermediary factors that are required in addition to general initiation factors for transcriptional activation has been reported in yeast, human, and *Drosophila* systems (19, 25, 35–38). Such factors, termed mediator, coactivator, USA, or transcriptional intermediary factor (TIF), have been viewed as various manifestations of a common activity involved in the activation process. It now appears, however, that mediator and HeLa coactivator may be distinguished in at least two respects: mediator supports effects of acidic activators, whereas HeLa coactivator does not, and mediator is readily separable from TFIID, whereas HeLa coactivator appears closely associated. Here we have demonstrated that the only additional factors required for activation with the Bio-Rex–DE52 fraction is purified recombinant TFIID and fraction c, for both *S. cerevisiae* and *Sch. pombe*. Further, we were unable to obtain activation in the Jurkat system upon addition of recombinant hTFIID (P.M.F., unpublished observations), probably due to the removal of the TAF proteins (37) associated with the endogenous hTFIID. These results indicate that the mechanism of activation in *S. cerevisiae* and *Sch. pombe* may differ from that found in higher eukaryotes. The characteristics of fraction c reported here suggest a relationship to USA. Both activities influence basal transcription and may be inhibitory as well as stimulatory. If mediator is truly distinct from both coactivator and USA, then the question arises of whether counterparts of yeast mediator may be identified in higher organisms. Indeed, TIF, which is required for transcriptional activation by GAL4-VP16 in the HeLa system, may represent such a factor.

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