Resolution of Factors Required for the Initiation of Transcription by Yeast RNA Polymerase II*

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Fractionation of a yeast nuclear extract reveals at least four factors required in addition to RNA polymerase II for accurate initiation of transcription. One of these factors can be replaced by HeLa transcription factor IID or by its yeast counterpart expressed in Escherichia coli. Each of the remaining three factors can be replaced by a fraction from yeast whole cell extract, facilitating further purification of the factors.

As many as five protein factors (for review, see Refs. 1 and 2) have been identified in mammalian extracts which are required in addition to RNA polymerase II (pol II) for initiation of transcription at a minimal promoter ("TATA" element plus transcription start site). Two of these factors from HeLa cells, termed TFIIID or BTF-3 and TFIIF, and also two factors from rat liver, referred to as α and β, have been purified to homogeneity (2–7). HeLa TFIIID, the factor that binds the TATA element, has proved difficult to enrich beyond about 250-fold (8). This difficulty prompted a search for a related activity in yeast extracts, culminating in the cloning and expression of a 27-kDa yeast polypeptide that can be substituted for TFIIID in the HeLa system (9–12). An activity that will replace TFIIIA has also been found in yeast extracts but not yet purified (13). Substitution of additional yeast factors will replace TFIIA has also been found in yeast extracts but may be enhanced by both yeast and mamalian gene activator proteins (15, 16).

Fractionation of Nuclear Extract—Nuclei were prepared and resuspended as described from 20 liters of Saccharomyces cerevisiae (14). Ammonium sulfate (3 M, pH 7.4) was added to a concentration of 0.5 M, and the suspension was kept for 30 min at 4 °C and centrifuged in a Beckman Ti-45 rotor at 40,000 rpm for 90 min at 4 °C. The supernatant (5–6 mg of protein/ml) was adjusted to 0.4 M ammonium sulfate with buffer A(0), and nucleic acids were removed by passage through a column of DEAE-Sepharose (50 ml) equilibrated with buffer A(400).Solid ammonium sulfate was added to 75% of saturation, and the precipitate was collected by centrifugation in a Beckman SW 28 rotor at 25,000 rpm for 25 min at 4 °C. The pellet (about 1 g of protein) was suspended in buffer A(0) to a protein concentration of 20 mg/ml and dialyzed against 1 liter of buffer A(0) until the conductivity was equivalent to that of buffer A(50). The dialysate was centrifuged in a Beckman SS 34 rotor at 10,000 rpm for 10 min at 4 °C. The supernatant (800 mg of protein) was applied to a DEAE column (50 ml) equilibrated with buffer A(50). The column was washed with 100 ml of buffer A(50) and was eluted with 150 ml of buffer A(120) and buffer A(350). The flow-through and wash (500 mg of protein), A(120) eluate (150 mg of protein), and A(350) eluate (150 mg of protein) were concentrated by the addition of solid ammonium sulfate and centrifugation as described above, and the pellets were suspended in buffer A(0) at protein concentrations of about 15 mg/ml.

The flow-through and wash were dialyzed against buffer A(0) to the conductivity of buffer A(40), passed through a P11 column (10 ml) equilibrated in buffer A(40), concentrated as described above, suspended in buffer A(0) at a protein concentration of 12 mg/ml, heated in 0.1-ml aliquots in 0.5-ml microcentrifuge tubes for 30 min at 50 °C, chilled for 10 min in ice, and centrifuged at 13,000 rpm for 8 min at 4 °C, to give fraction c.

The A(120) eluate of the DEA column was dialyzed against buffer A(0) to the conductivity of buffer A(50), diluted in buffer A containing 150 mM potassium acetate to a protein concentration of 1 mg/ml, heated in 50-μl aliquots for 7 min at 47 °C, and chilled for 10 min in ice, to give fraction c.

The A(350) eluate of the DEA column was dialyzed and passed through P11 as described above for the flow-through and wash to give fraction d.

The preparation of fractions b and d, nuclear extract was passed through DEAE-Sepharose, concentrated, suspended, dialyzed, and centrifuged as described above, except that dialysis was to the conductivity of buffer A containing 0.07 M KCl. The material was applied to a P11 column (10 ml) equilibrated in buffer A containing 0.07 M KCl, and the column was developed with a gradient of buffer A containing 0.07–0.7 M KCl. The peak of fraction c activity was at about 0.33 M KCl, and that of fraction d was at about 0.44 M KCl. All fractions...
were frozen in liquid nitrogen and were stable for several months when stored at −80°C.

Fractionation of Whole Cell Extract—The preparation of whole cell extract was from 12 liters of culture as described (17) except that no KCl was added before bead beating, and ammonium sulfate (3 M, pH 7.4) was added immediately after to a concentration of 0.4 M. Following centrifugation as described (17), the clarified extract was passed through a DE52 column (100 ml) equilibrated in buffer A(400) to remove nucleic acids. One-quarter of the material (1.2 g of protein) was then dialyzed to the conductivity of buffer A(50) and applied to a DEAE column (100 ml) equilibrated with buffer A(50). The column was washed with 200 ml of buffer A(50) (750 mg of protein in the flow-through and wash) and eluted with 300 ml each of buffer A(120) (180 mg of protein, designated WCE 120) and buffer A(350) (200 mg of protein, designated WCE 350).

Preparation of Yeast TFIID—An extract was prepared from a strain of Escherichia coli expressing yeast TFIID as described (11). The extract was diluted to a protein concentration of 5 mg/ml with buffer B, adjusted to 0.15% (w/v) polyethyleneimine by the addition of 10% polyethyleneimine in buffer B dropwise with stirring at 4°C, stirred a further 2 h, and centrifuged in a Beckman JA-20 rotor at 15,000 rpm for 30 min at 4°C. The pellet was stirred with a volume of 0.3 M ammonium sulfate in buffer B equal to half that of the supernatant for 30 min at 4°C and centrifuged as before. The supernatant was brought to 50% of saturation with solid ammonium sulfate, stirred for 20 min at 4°C, and centrifuged as before. The pellet was suspended in buffer A(0) and dialyzed against buffer A(0) containing 100 mM potassium acetate. The dialysate (4.3 mg of protein/ml) was stored at −80°C.

Transcription Assays—Templates for transcription were pCG-,

RESULTS AND DISCUSSION

Yeast nuclear extract prepared as described (14) was initially divided into three fractions on a DEAE-Sepharose column (Fig. 1), one that flowed through in 0.05 M ammonium sulfate and others that bound and were eluted with 0.12 and 0.35 M ammonium sulfate. The flow-through was freed of some inhibitory components and enriched by passage through phosphocellulose in 0.04 M ammonium sulfate and by heating for 30 min at 50°C to give fraction c. The 0.35 M eluate of the DEAE column was passed through phosphocellulose in 0.04 M ammonium sulfate to give fraction a. Fractions a and c could be combined with the 0.12 M eluate of the DEAE column to reconstitute transcriptional activity (not shown), but on heating the 0.12 M eluate for 7 min at 47°C (to give fraction e), two additional fractions were required. These additional fractions (b and d) could be obtained by chromatography of nuclear extract on phosphocellulose, without contamination by fractions a and c, which flowed through, or by fraction e, which was unstable under the conditions of the chromatography.

When fractions a–e were combined, full transcripational activity was obtained (Fig. 2). Transcription was initiated at two sites in a yeast CYCI promoter/G-minus cassette construct, as found with nuclear extract and as observed in vivo (15). The recovery of activity was 80–140% from the nuclear extract, presumably reflecting the removal of inhibitors. Transcription was absolutely dependent on all five fractions (less than 5% of the level for the complete system when any fraction was omitted). Some of the fractions supplied pol II, whose involvement was shown by inhibition by α-amanitin at 10 μg/ml. All of the fractions contained additional essential factors since none could be replaced by purified yeast pol II known to be active in the initiation of transcription (18). As mentioned above, an activity has been purified from yeast (yIID) that will substitute for human TFIID (hIID) in the HeLa transcription system. The location of the transcription start site, approximately 30 base pairs downstream of the TATA element, is unaffected by this substitution (9–12). Conversely, we find that hIID can replace fraction d in the yeast system (Fig. 3). The location of the transcription start sites, 63–69 base pairs downstream of the TATA element of the adenoviral major late promoter in the yeast system, is unaffected by the substitution. We conclude that fraction d contains a TATA-binding factor and that additional components of the system determine the location of the initiation site.

Fraction a may correspond with the activity identified previously in yeast extracts that can substitute for TFIIA in the HeLa system (13). Both fraction a and yeast TFIIF bind tightly to DEAE-resins and fail to bind to anionic resins.

Preliminary results suggest that fraction c becomes dispensable when the other fractions are purified further. The effect

FIG. 1. Derivation of fractions from nuclear extract required for the initiation of transcription by yeast RNA polymerase II.

FIG. 2. Requirement of fractions from nuclear extract for the initiation of transcription by yeast RNA polymerase II. The complete reaction contained fractions a (5 μg), b (3 μg), c (4 μg), d (2 μg), e (10 μg), and pCG- as template. Fractions were omitted or α-amanitin added (10 μg/ml) as indicated.

FIG. 3. Substitution of yeast TFIIID and HeLa TFIIID for fraction d in the transcription assay. The complete reaction (+d) was as in Fig. 2 except with pMLCG- as template. Fraction d was omitted from the remaining reactions and yeast TFIIID (yIID, 8 μg) or HeLa TFIIID (hIID, 1 μg) added as indicated.

*P. Flanagan, unpublished observations.
FIG. 4. Substitution of fractions from whole cell extract for those from nuclear extract in the transcription assay. The complete reaction was as in Fig. 2. Fractions were omitted and fractions from whole cell extract added (WCE 120, 15 µg; WCE 350, 12 µg) as indicated.

of fraction c is unusual in that no radioactivity is detectable in a gel either in bands or in the background when this fraction is omitted (Fig. 2). The activity in fraction c appears to be proteinaceous since it is thermolabile (inactivated by heating for 30 min at 55 °C) and it precipitates with the bulk of the protein in a nuclear extract (50% precipitated at 50% of saturation with ammonium sulfate). The activity may be a nuclease inhibitor or play some other nonspecific role.

Inasmuch as fraction d can be replaced by YIID and purified yeast pol II active in initiation is available, we are left with fractions a, b, and e that must be purified to arrive at a fully defined transcription system. This undertaking should be somewhat simplified by the finding that fractions a, b, and e can all be obtained from whole cell extract, which is easier to prepare than nuclear extract, especially on a large scale. Whole cell extract was divided into fractions eluting from DEAE-Sephacel with 0.12 M (WCE 120) or 0.35 M ammonium sulfate (WCE 350). WCE 120 could be substituted for either fraction b or fraction e in the transcription reaction with other fractions from nuclear extract as described above (Fig. 4). Similarly, WCE 350 could be substituted for fraction a. Inclusion of both WCE 120 and WCE 350 in the reaction gave a much diminished transcription signal, presumably due to inhibitors derived from the whole cell extract (data not shown). The yields of fraction a, b, and e activities in WCE 120 and WCE 350 were comparable to (about 40% of) those obtained from nuclear extract, but the specific activities were 5–10-fold lower.

The fractionation of nuclear extract reported here gives a minimal estimate of the number of protein factors required in addition to RNA polymerase for initiation of transcription at a minimal pol II promoter in yeast. The importance of the fractions from nuclear extract may lie principally in their use for assay of factors from whole cell extract, facilitating the purification of these factors to homogeneity in amounts sufficient for biochemical and structural studies.

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