Quantitation of the RNA Polymerase II Transcription Machinery in Yeast*

Tilmann Borggreve‡, Ralph Davis‡, Avital Bareket-Samishh,§ and Roger D. Kornberg|

From the Department of Structural Biology, Stanford University School of Medicine, Stanford, California 94303

Received for publication, October 4, 2001
Published, JBC Papers in Press, October 8, 2001, DOI 10.1074/jbc.M109581200

TAP tags and dot blot analysis have been used to measure the amounts of RNA polymerase II transcription proteins in crude yeast extracts. The measurements showed comparable amounts of RNA polymerase II, TFIIE, and TFIIF, lower levels of TBP and TFIIB, and still lower levels of Mediator and TFIH. These findings are consistent with the presumed roles of the transcription proteins, but do not support the idea of their recruitment in a single large complex to RNA polymerase II promoters. The approach employed here can be readily extended to quantitative analysis of the entire yeast proteome.

Seven pure proteins are required for promoter-dependent, regulated RNA polymerase II transcription: TFIIB, TFIID, TFIIE, TFIIF, TFIIH, Mediator, and the polymerase itself (1–4). All seven proteins are thought to assemble in a giant complex at a promoter prior to the initiation of transcription. Many of the proteins interact in solution, for example, TFIIB with TFIIA, TFIIE with TFIIH, and TFIIF and Mediator with RNA polymerase II. Indeed it has been proposed that all the proteins except TFIID and TFIIE are recruited to a promoter as a preassembled complex (5). While a test of this proposal is defrayed in part by the payment of page charges. This article must advertised in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of postdoctoral fellowships from the Deutsche Forschungsgemeinschaft and from the Schweizer Nationalfonds.
‡ These two authors contributed equally to this work.
¶ To whom correspondence should be addressed. Tel.: 650-723-6988; Fax: 650-723-8464; E-mail: kornberg@stanford.edu.

MATERIALS AND METHODS

GST*-TAP—Primers GST-TAP forward and GST-TAP reverse (GST-TAP forward: CGTGCTCTCGACATCATTACATCGTTGACTCCCGCGGA; and TAP-reverse: GTGTGGCTTCGACGCGTACATCGTTGACTCCCGCGGA) were used to amplify the TAP tag region of pBS1479 obtained from the Seraphin laboratory (12). The resulting PCR fragment was digested with BamHII and cloned into pGEX6P1 (Pharmacia Corp.). Resulting clones were confirmed by restriction digestion and immunoblotting in BL21 cells.

The GST-TAP construct in BL21 cells was grown at 37 °C to an A600 of 0.8 and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 25 °C. Cells were washed in PBS and frozen. Thawed cells were sonicated and centrifuged. Clarified extract was applied to a glutathione affinity column (Sigma), washed with PBS, and eluted with PBS + 15 mM glutathione (Sigma). The GST-TAP fusion protein was further purified by electroelution using an Elutrap-Electrophoresis Chamber (Schleicher and Schuell). The resulting GST-TAP was pure as judged from 10% SDS-polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue.

TAP-tagged Strains—TAP-tagged strains were prepared as described in Refs. 12, 22. In brief, PCR was performed with forward and reverse primer pairs using pBS1479 as the template; pBS1479 contains the TAP tag cassette followed by a trp + selection marker. The resulting PCR product was used to transform yeast strain CB010 (MATa, pep4::HIS3Ipr1::LEU2, pr1::1-HISG, can1, ade2, trpl, ura3, his3, leu2-3,112) as described in (23). Transformants (Table I) were screened by PCR, and positives were confirmed by immunoblotting as described below.

Cell Growth—Cells were grown in minimal media lacking tryptophan, as described (24) or on fhcerc.org/labs/gottschling/hc.sh, to mid-log phase, [A600] = 0.5, or 2 × 10^6 cells). Cells were harvested, washed once with water, and counted using an Improved Neubauer Brightline Hemocytometer (Hausser Scientific).

Cell Extract—Cells (10^7) were washed once with water and resuspended in 30 μl of 2% SDS, and 100 μl of glass beads were added. Cells were subjected three times to 2 min of boiling and 2 min of vortexing. Then 300 μl of 1 M NaCl/0.5 M urea/PBS were added, followed by vortexing and centrifugation at 13,000 rpm for 1 min. The clarified supernatant (10 μg and subsequent dilutions) was used for the dot blot assay.

Protein concentrations were determined in triplicate with the Bradford reagent (13), and the amounts of cell extracts applied to dot blots were adjusted accordingly. Protein quality was verified by immunoblotting. For this purpose, the extracts were run on a SDS-10% polyacrylamide gel, transferred to an Immobilon-P membrane (Millipore), and probed with anti-RPB3 (1Y26) and antiRpb1 (8W16) antibodies. Detection was performed with horseradish peroxidase-conjugated goat-anti-mouse antibodies (Bio-Rad) and ECL (Amersham Pharmacia Biotech).

§ The abbreviations used are: GST, glutathione S-transferase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; TBP, TATA-binding protein.
RESULTS

Quantitation of transcription proteins in yeast cell extracts has been performed in the past by immunoblot analysis. It has been limited to the few polypeptides for which high quality polyclonal antibodies are available. Most antibodies produce unacceptably high cross-reactivity and background signals on blots from crude lysates. We circumvented this problem by taking advantage of the two protein A modules within the TAP tag (12). The affinity and specificity of protein A-IgG interaction proves to be sufficient for detection of even very scarce proteins in crude extracts with no background at all.

TAP tags were introduced at the C termini of transcription proteins by integration at genomic loci. TAP-tagged strains were checked by PCR and immunoblotting to ensure correct integration (Fig. 1).

To measure the absolute amounts of tagged proteins, an external standard was prepared. A TAP tag was introduced at the C terminus of Escherichia coli GST. The resulting GST-TAP was purified to homogeneity and was quantitated by Bradford assay (13).

Dot Blot Assay — Extraction of proteins from TAP-tagged strains was accomplished by bead beating and boiling in the presence of SDS. Optimal extraction of transcription proteins was obtained with the inclusion of 1 M NaCl and 2 M urea in the solubilization buffer. The number of cells extracted could be varied by an order of magnitude or more without effect on the results. Dilution series were prepared in 96-well plates, along with dilution series of GST-TAP and of a negative control (extract of the untagged, parental wild-type strain). Following transfer from a 96-well plate to a polyvinylidene difluoride-membrane with a 96-well vacuum dot-blotter, the membrane was washed, blocked (14), and subjected to a one-step immunoaffinity procedure with an anti-peroxidase-IgG conjugate (15). Bound IgG was detected by chemiluminescence (Fig. 2 A), followed by scanning (Umax Astra4000U) and quantitation (ImageQuant). As little as 1 fmol of protein could be detected, corresponding to 500 molecules per cell in a sample prepared from 10⁶ cells. This detection limit allows the quantitation of proteins present at very low levels and the processing of many samples rapidly and in parallel.

Quantitation of the RNA Polymerase II Transcription Machinery — Yeast strains bearing TAP tags on various transcription proteins were grown to mid-log phase in minimal medium and harvested at the same cell density. Quantitation was performed by dot blot analysis as described, with all measurements in triplicate, resulting in standard deviations of 10–20%.

Three observations indicated that the presence of the TAP tag did not alter the expression level of the protein under study. First, growth curves of all strains were determined in rich and in minimal media, and no significant variation from the wild-

### Table I

| Strain       | Genotype                                                                 |
|--------------|---------------------------------------------------------------------------|
| CB010        | Mata, pep4 : HIS3/prb1 : LEU2, prc1 : HISG, can1, ade2, trp1, ura3, his3, leu2-3, 112 |
| Med8-TAP     | identical to CB010 except Med8 : Med8-TAP-Trp                             |
| Rgr1-TAP     | identical to CB010 except Rgr1 : Rgr1-TAP-Trp                             |
| Med7-TAP     | identical to CB010 except Med7 : Med7-TAP-Trp                             |
| Rpβ3-TAP     | identical to CB010 except Rpβ3 : Rpβ3-TAP-Trp                             |
| Tfa2-TAP     | identical to CB010 except Tfa2 : Tfa2-TAP-Trp                             |
| Suα7-TAP     | identical to CB010 except Suα7 : Suα7-TAP-Trp                             |
| TBP-TAP      | identical to CB010 except TBP : TBP-TAP-Trp                               |
| Tfg2-TAP     | identical to CB010 except Tfg2 : Tfg2-TAP-Trp                             |
| Tfb3-TAP     | identical to CB010 except Tfb3 : Tfb3-TAP-Trp                             |
| Sd2-TAP      | identical to CB010 except Sd2 : Sd2-TAP-Trp                               |
| Tfb4-TAP     | identical to CB010 except Tfb4 : Tfb4-TAP-Trp                             |
| Ccl1-TAP     | identical to CB010 except Ccl1 : Ccl1-TAP-Trp                             |

**Fig. 1. Immunoblot of extracts from TAP-tagged strains probed with rabbit anti-peroxidase IgG.** Crude extracts (5 μg) from strains bearing TAP tags on the polypeptides indicated were analyzed.
type strain was observed (data not shown). As the tagged strains were haploid and many of the tagged components are essential for viability, a decrease in expression level should have been manifested by growth retardation. Second, independent measurements of the levels of three Mediator subunits gave essentially the same results, as did measurements on four TFIIH subunits (Fig. 2B). Moreover, the levels of tagged subunits of Mediator and TFIIH complexes judged from intensities of Coomassie staining in SDS-gels were essentially the same as those of untagged subunits (not shown). Finally, immunoblots with monoclonal antibodies against RNA polymerase II subunits Rpb3 and wild-type (CB010) strains were prepared and blotted as described.

The results (Table II) showed the greatest abundance of RNA polymerase II, in keeping with its involvement in both the initiation and elongation of transcription. The level of TFIIF was nearly the same, as expected from its tight association with RNA polymerase II and from its likely role in both transcription initiation and elongation as well (16). The levels of Mediator and TFIIH were much lower, consistent with their involvement only in transcription initiation and only transiently in complexes with RNA polymerase II. It was surprising to find an amount of TFIIE comparable with that of TFIIF.
rather than TFIIH, since TFIIIE interacts with TFIIH and is believed to be responsible for its recruitment to transcription initiation complexes. Finally, TBP and TFIIIB were present in equal amounts, intermediate between those of RNA polymerase II and Mediator/TFIIH. This finding is inconsistent with a previous report of TBP and TFIIIB levels 2- to 3-fold greater than that of RNA polymerase II (17), perhaps due to differences in the methods of extract preparation or quantitation. Our result is, however, entirely consistent with other information about the roles of TBP and TFIIIB. The two proteins form a complex at the TATA box of RNA polymerase II promoters and so should be present in equal amounts. They are only involved in initiation and so should occur at a lower level than the polymerase. They may persist at a promoter through multiple rounds of transcription, accounting for their occurrence at a higher level than TFIIH or Mediator.

DISCUSSION

The method of protein quantitation presented here, based on TAP-tagging and dot blot assay, is rapid and sensitive. In the example described, it was applied to the measurement of protein levels in crude cell extracts. It can also be of great utility for monitoring protein purification at early stages where protein levels are below the detection limit of polyclonal antibodies or where such antibodies are not available. The method can readily be applied on a genome-wide scale to quantitate the entire yeast proteome. It is preferable to two-dimensional gel electrophoresis for this purpose for cases of very small or very large proteins or those that occur at very low levels. Once TAP tags have been introduced they offer the further advantage of facile isolation for biochemical analysis.

Our measurements of the levels of RNA polymerase II transcription proteins do not support the hypothesis of recruitment to a promoter in the form of a single large complex. Rather they are consistent with distinct modes of entry, such as persistent association of TBP and TFIIIB with the promoter and transient interaction of Mediator and TFIIH. As Mediator and TFIIH are the least abundant components of the transcription machinery, they may be the most susceptible to regulation. Indeed, Mediator is the target of many activators and repressors (4) while TFIIH, containing kinase and helicase activities, plays a pivotal role in promoter opening and clearance. The protein kinase activity of TFIIH is regulated by Mediator in vitro (18) and TFIIIE (19), and its action on the C-terminal domain of RNA polymerase II is tightly regulated in vivo (20).

Although TFIIH function in transcription initiation and promoter clearance is dependent on TFIIE, the level of TFIIE is severalfold greater, comparable with the levels of TFIIIF and RNA polymerase II. This raises the possibility that TFIIE is involved in the elongation as well as the initiation of transcription. Alternatively, TFIIE might be subject to rapid turnover or to inactivation by posttranslational modification. Indeed, acetylation of TFIIE has been reported in the human system (21).

REFERENCES

1. Kornberg, R. D. (1998) Cold Spring Harb. Symp. Quant. Biol. 63, 229–232
2. Orphanides, G., Lagrange, T., and Reinberg, D. (1996) Genes Dev. 10, 2657–2683
3. Lee, T. I., and Young, R. A. (2000) Annu. Rev. Genet. 34, 77–137
4. Myers, L. C., and Kornberg, R. D. (2000) Annu. Rev. Biochem. 69, 729–749
5. Kolessa, A. J., and Young, R. A. (1995) Trends Biochem. Sci. 20, 113–116
6. Shalon, D., Smith, S. J., and Brown, P. O. (1996) Genome Res. 6, 639–645
7. Lashkari, D. A., DeRisi, J. L., McCusker, J. H., Namath, A. F., Gentile, C., Hwang, S. Y., Brown, P. O., and Davis, R. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13057–13062
8. Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999) Mol. Cell. Biol. 19, 1720–1730
9. Pradet-Balade, B., Boulme, F., Beug, H., Mulliner, E. W., and Garcia-Sanz, J. A. (2001) Trends Biochem. Sci. 26, 225–229
10. Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Boucherie, H., and Mann, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14440–14445
11. Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Nat. Biotechnol. 17, 994–999
12. Righut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999) Nat. Biotechnol. 17, 1030–1032
13. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
14. Maniatis, T., Fritsch, E. F., Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Sternberger, L. A., Hardy, P. H., Jr., Cuculis, J. J., and Meyer, H. G. (1970) J. Histochem. Cytochem. 18, 315–333
16. Conaway, J. W., Shilatifard, A., Dvir, A., and Conaway, R. C. (2000) Trends Biochem. Sci. 25, 375–380
17. Lee, T. I., and Young, R. A. (1998) Genes Dev. 12, 1398–1408
18. Kim, Y. J., Bjorklund, S., Li, Y., Sayre, M. H., and Kornberg, R. D. (1994) Cell 77, 599–608
19. Ohkuma, Y., and Roeder, R. G. (1994) Nature 368, 160–163
20. Dahmus, M. E. (1985) Biochim. Biophys. Acts 261, 171–182
21. Ihn, A., Yang, X. J., Ogryzko, V. V., Nakatan, Y., Wolffe, A. P., and Ge, H. (1997) Curr. Biol. 7, 689–692
22. Puig, O., Rutz, B., Luukkonen, B. G., Kandel-Lewis, S., Bragado-Nilsson, E., and Seraphin, B. (1998) Yeast 14, 1139–1146
23. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
24. Sherman, F. (1991) Methods Enzymol. 194, 3–21
