Factors Involved in Specific Transcription by Mammalian RNA Polymerase II

RNA POLYMERASE II-ASSOCIATING PROTEIN 30 IS AN ESSENTIAL COMPONENT OF TRANSCRIPTION FACTOR IIF*

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Transcription from class II promoters requires five general factors, IIA, IIB, IID, IIE, and IIF, in addition to RNA polymerase II for basal levels of transcription (Reinberg, D., Flores, O., and Buckbinder, L. (1987) in Molecular Biology of RNA: New Perspectives (Inouye, M., and Dudock, B., eds) pp. 423-439, Academic Press, Orlando, FL). A protein fraction containing transcription factors (TF) IIE and IIF was able to reconstitute transcription from the adenovirus major late promoter when added to extracts depleted of the RNA polymerase II-associating proteins RAP 30 and RAP 74 (Sopta, M., Carthew, R. W., and Greenblatt, J. (1985) J. Biol. Chem. 260, 10353-10360). Studies with monoaffinity-purified antibodies directed against RAP 30 demonstrated, by Western blot analysis, that RAP 30 copurifies on five columns with transcription factor IIF. That RAP 30 is a functional component of TFIIIF was also demonstrated; preincubation of anti-RAP 30 antibodies with purified TFIIIF inhibited transcription. Inhibition of transcription was overcome by the addition of purified TFIIIF. RAP 30 is an integral part of a preinitiation complex; the incubation of all the general transcription factors with a promoter-containing DNA resulted in a complex that was not inhibited by anti-RAP antibodies. Incubation of the transcription factors in the absence of a promoter-containing DNA resulted in a complex that was partially resistant to the antibodies.

Several soluble cell-free systems that allow accurate initiation of transcription by RNA polymerase II on exogenously added DNA templates have been developed (1-3). The fractionation of these extracts has led to the identification of several factors required for transcription of class II promoters (4-9). We have identified five general factors, TFIIA, -IIB, -IID, -IIE, and -IIF, that operate via the TATA sequence and were required, in addition to RNA polymerase II, for basal levels of transcription (10). TFIIID is a specific DNA binding protein that binds to the TATA box element (11). TFIIA and TFIIID are required for the formation of the committed complex at the promoter (9). The transcriptional activity of factors IIB, IIE, and IIF appears to be mediated via protein-protein interactions. Reinberg and Roeder (8) have shown that a preparation containing TFIIE and TFIIIF could be made to co-sediment with RNA polymerase II and with TFIIIB.

Other groups have also reported the isolation of factors required for transcription of class II promoters. Zheng et al. (12) have isolated a 27,000-dalton protein (BTF3) from HeLa cells and demonstrated that purified BTF3 co-sedimented with RNA polymerase II. Sopta et al. (13), utilizing RNA polymerase II affinity columns, have isolated three RNA polymerase II-associating proteins, RAP 30, RAP 38, and RAP 74. Burton et al. (14) showed that at least one of these RAPs was required for accurate initiation of transcription in vitro. RAP 38 was shown not to be required for transcription initiation but seemed to be an elongation factor (14, 18) similar to IIIS (15) and SII (16, 17). Furthermore, Burton et al. (18, 19) showed that monoaffinity-purified antibodies against RAP 30 inhibited transcription when added to HeLa cell nuclear extracts and that RAP 50 is a generally required initiation factor. It was also demonstrated that a complex of RAP 30 and RAP 74 was required to restore accurate transcriptional activity to an extract depleted of these factors by immunoprecipitation with anti-RAP 30 antibodies (19).

In this article we demonstrated that TFIIIE, a newly identified factor purified from the previously described preparation of TFIIIE (8), contains RAP 30 as an essential component. The prior incubation of purified TFIIIF with monoaffinity-purified anti-RAP 30 antibodies before addition to a reaction mixture containing purified components abolished transcription from the adenovirus major late promoter (Ad-MLP). Incubation of TFIIIF with the other general transcription factors, RNA polymerase II and a promoter-containing DNA, led to the formation of a transcription-competent DNA-protein complex that was not inhibited by anti-RAP antibodies.

The abbreviations used are: TF, transcription factor; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RAP, RNA polymerase II-associating protein; Ad-MLP, adenovirus major late promoter; MLP, major late promoter.

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MATERIALS AND METHODS

The purification of the transcription factors was as previously described (8). RAP 30-74-depleted extract was prepared by immuno-purification of the anti-RAP 30 antibodies and protein A-Sepharose beads, as previously described (19). The affinity purification of the anti-RAP 30 antibodies and the Western blots were as previously described (19).

Transcription reactions (40 μl) were performed in a buffer containing 40 mM Hepes (pH 7.9), 12% glycerol, 55 mM KCl, 10 mM ammonium sulfate, 2% polyethylene glycol 8000, 5 mM dithiothreitol, 0.6 mM ATP and CTP, 0.025 mM [32P]UTP, and template DNA as indicated in the figure legends. The DNA was pML(CAT) (20). The adenovirus major late promoter directed transcription from a 380-base pair synthetic polynucleotide chain which lacks CMP residues in the transcribed DNA strand.

RESULTS AND DISCUSSION

Five General Factors Are Required for Transcription from the MLP—We and others have documented that transcription from the adenovirus major late promoter required at least four protein fractions in addition to RNA polymerase II (5–8, 10, 20). These fractions contained factors (TFIIA, -IIB, -IID, and -IIE) that operated via the TATA sequence. Further fractionation of the preparation of TFIIE resulted in the identification of a new transcription factor, TFIIF (Ref. 10; see Fig. 3A). TFIIF was absolutely required for transcription; the omission of TFIIF from an assay reconstituted with purified factors and DNA carrying the Ad-MLP resulted in no detectable transcription (Fig. 1, lane 7). Furthermore, the addition of TFIIF did not obviate the requirements for the general factors or the upstream specific factor MLTF (11, 22) (Fig. 1).

Extracts Depleted of RAP 30-74 Can Be Complemented for Transcription from the Ad-MLP by Transcription Factors IIE and IIF—Previous studies have demonstrated that the factor responsible for transcriptionally active extracts by chromatography on RNA polymerase II affinity columns resulted in the binding of three major polypeptides (RAP 30, 38, and 74) to the column (13) and the production of transcriptionally inactive extracts (break-through fractions) (14). However, when the flow-through fractions were supplemented with the RAP mixture they regained transcriptional activity (14). Removal of RAP 30 and RAP 74 from an extract as a RAP 30-74 complex with antibody to RAP 30 also inhibited the extract (19). In this case the depleted extract could be fully reconstituted with electrophoretically purified RAP 30 and RAP 74 (19). Reinberg and Roeder (8) demonstrated that the previously described preparation of TFIIE contained at least one transcription factor that bound to purified RNA polymerase II and TFIIB. On the basis of these results, we investigated whether the previously described TFIIB and/or TFIIE protein fractions were able to complement an RAP 30-74-depleted extract for transcription from the Ad-MLP. The addition of a DNA template containing the Ad-MLP to RAP 30-74-depleted extract did not result in the generation of detectable levels of transcription (Fig. 2, lane 1). However, addition of a preparation of TFIIE (containing TFIIF, see below) and a promoter containing DNA resulted in low but specific levels of transcription (Fig. 2, lane 3). The TFIIB protein fraction was unable to complement the RAP-depleted extract (lane 2).

The addition of the phosphocellulose 0.5 M fraction (containing TFIIB, TFIIE, and TFIIF, see Fig. 3A) resulted in transcription; however, the levels of activity were similar to those obtained when only the preparation of TFIIE was added to the depleted extract. These results suggested that the depleted extract contained TFIIB but was deficient in TFIIE and/or TFIIF. The low levels of transcriptional activity observed with the depleted extract, in comparison to those obtained with a system reconstituted with partially purified general transcription factors (compare lanes 3 and 5), could result from limiting IIA and/or IID, possible as a result of its partial removal by co-precipitation by the anti-RAP 30 antibodies (see below).

Inactivation of TFIIF Activity by Antibodies Directed against RAP 30—The preparation of TFIIF containing TFIIF activity was able to partially complement the transcriptional activity of an RAP 30-74-depleted extract. Furthermore, addition of TFIIB had no effect on the transcriptional activity of the depleted extract. These results are in agreement with those of Reinberg and Roeder (8), which demonstrated that a partially purified preparation of TFIIE interacted with RNA polymerase II and that no stable complex was formed between TFIIB and the polymerase. However, we (8) and others (12, 19) have suggested that RAP 30 might be equivalent to TFIIB. This was suggested by the observation that upon purification of TFIIB to near homogeneity (8), the activity coeluted, on
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glycerol gradient sedimentation and gel filtration, with a polypeptide of 30,000 daltons. Therefore, TFIIB has a molecular weight similar to that of RAP 30. In order to further investigate whether RAP 30 corresponded to TFIIB, TFIIE, or TFIIF, different fractions from the purification of these factors (see. Fig. 3A) were analyzed by Western blots using antibodies against RAP 30 (19). The anti-RAP 30 was more specific because it was affinity-purified on a column containing an immobilized RAP 30-β-galactosidase fusion protein produced in Escherichia coli (19). These analyses indicated that the nuclear extract (Fig. 3B, lane 1) and the 0.5 M phosphocellulose fraction (lane 2) contained a protein of approximately 30,000 daltons that reacted with the antibodies (see figure legend for details). Further fractionation of the phosphocellulose fraction by DEAE-cellulose column chromatography resulted in two fractions, IIB and IIE (see Fig. 3A), required to reconstitute maximal levels of transcription (8, 20, 21). When equivalent amounts of transcriptional activity of different IIB-DEAE-cellulose protein fractions were used in the Western blot analysis, variable amounts of RAP 30-reacting material were observed (lanes 3–6). However, when more highly purified fractions of IIB were used, no RAP 30-reacting material was observed (lanes 7–9). A similar analysis utilizing the IIE fractions indicated that through the Affi-Gel blue chromatographic step (see. Fig. 3A) all contained equal amounts of RAP 30-reacting material (lanes 12–14). Additional purification of the IIE-Affi-Gel blue protein fraction by chromatography on a high pressure liquid chromatography DEAE-5PW column resulted in the separation of two factors, TFIIE and TFIIF, both required to reconstitute transcription (see Fig. 1). Analysis of the Western blot indicated that the TFIIF, but not the TFIIE fraction, reacted with the anti-RAP 30 antibodies (lanes 11 and 10, respectively). These suggested that TFIIF corresponded to RAP 30. The presence of variable amounts of RAP 30-reacting material in the IIB-DEAE-cellulose protein fraction is in agreement with previous observations that indicated a copurification of variable amounts of TFIIE with TFIIF (8) and with the demonstration that a partially purified TFIIE protein fraction (containing TFIIF) interacted with TFIIB (8).

It has previously been shown that RAP 30 is required for accurate initiation in a crude transcription system (14, 19). To test whether RAP 30 is also required for initiation in a transcription system reconstituted with purified factors, increasing amounts of the monoaflinity-purified anti-RAP 30 antibodies were incubated with a constant amount of TFIIF prior to the addition of TFIIF to mixtures containing the DNA template and the other purified factors. This resulted in an inhibition of transcription (Fig. 4A, lanes 1 and 2). The observed inhibition was specific for the anti-RAP 30 antibodies; preincubation of TFIIF with antibodies directed against β-galactosidase had no effect on transcription (lanes 3 and 4). In order to directly show that RAP 30 is a functional component of TFIIF activity, the experiment outline in the lower part of Fig. 4B was performed. A fraction containing TFIIE and TFIIF was incubated with antibodies (directed against RAP 30 or β-galactosidase) for 20 min. Protein A-Sepharose saturated with bovine serum albumin was then added and the antibodies bound to protein A removed by centrifugation. Aliquots of the aqueous phase were added in place of IIE and TFIIF to transcription reaction mixtures. The result of this experiment (Fig. 4B) indicated that incubation of TFIIE and TFIIF with anti-RAP 30 antibodies prevented complementation (lanes 3 and 4). This inhibition was specific for the anti-RAP 30 antibodies; the incubation of the TFIIE and TFIIF with antibodies directed against β-galactosidase had no effect on transcription (lanes 5 and 6). The observed inhibition by the anti-RAP 30 antibodies was overcome by the addition of purified TFIIF (devoid of TFIIE) (lanes 7 and 8); however, the addition of purified TFIIF (devoid of TFIIF) did not overcome this inhibition (lanes 9 and 10). Furthermore, the incubation of TFIIF with anti-RAP 30 antibodies had no effect on transcription (data not shown). These results demonstrated that anti-RAP 30 antibodies inhibited transcription by specifically interacting with TFIIF. TFIIF/RAP 30 Is an Integral Part of the Preinitiation Complex—Incubation of TFIIF with antibodies directed against RAP 30 resulted in an inhibition of transcription. Based on this observation, we next analyzed whether the incubation, prior to the addition of the antibodies, of TFIIF with other transcription factors and/or a promoter-containing DNA would prevent this inhibition of transcription. Incubation of all the transcription factors and a promoter-containing DNA prior to the addition of the antibodies (RAP 30 or β-galactosidase) yielded similar amounts of transcription (Fig. 5A, lanes 6 and 7) as in the absence of antibody (lane 1). This result suggests that the formation of a protein-DNA complex sequestered TFIIF from inhibition by the anti-RAP 30 anti-
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Antibodies at phase were added as a source of TFIIE and TFIIF to the reaction mixtures.

The studies presented here indicate that RAP 30, a protein isolated using affinity chromatography on RNA polymerase II columns (13), is a functional component of TFIIF, a newly discovered general transcription factor. This conclusion was based on the following observations: (i) the TFIIF:RAP 30 ratio was constant during purification through five chromatographic steps (Fig. 3B); (ii) the incubation of TFIIF with monoaflinity-purified anti-RAP 30 antibodies inhibited transcription; (iii) a preparation containing TFIIF and TFIIE complemented the transcriptional activity of RAP 30-depleted extracts (9). It has previously been demonstrated that preparations of TFIIF (8) (containing TFIIF), but not TFIIB, were able to interact with purified RNA polymerase II. The studies presented here support this observation, namely that the preparation of TFIIF (containing TFIIF) was able to complement the transcriptional activity of an extract depleted of RAP 30-74 (13). Further investigations will determine whether purified TFIIF and/or purified TFIIF interacts

Fig. 4. Anti-RAP 30 antibodies (Ab) inhibited transcription from the MLP. A, reaction conditions were as described in the legend to Fig. 1. However, TFIIF (0.4 μg) was first incubated with antibodies at 4°C for 20 min prior to the addition into the transcription reaction mixtures. B, reaction mixtures were as described in the legend to Fig. 1. However, a fraction containing TFIIE and TFIIF (Sephacryl AcA 44, 17 μg) was first incubated with antibodies (anti-RAP 30 or β-galactosidase), as described in the panel at the bottom of the figure. Protein A bound to Sepharose (20 μg) was then added, and antibody-protein complexes were removed by centrifugation on a tabletop centrifuge for 5 min at 10,000 rpm. Aliquots of the aqueous phase were added as a source of TFIIE and TFIIF (lanes 3–12). In addition purified TFIIE (DEAE-5PW fraction, 0.35 μg, lanes 1, 7, 8, 11, 12) or purified TFIIE (DEAE-5PW fraction, 0.2 μg, lanes 1, 9–12) was also added. Reaction products were analyzed as described in the legend to Fig. 1.

bodies or that TFIIF activity, after a preincubation with the factors, was no longer required. The formation of this complex was dependent on incubation of the transcription factors and DNA. When the antibodies directed against RAP 30 were added together with the transcription factors and the MLP to the reaction mixtures, transcription was significantly reuced (compare lanes 4 and 5). The preincubation of purified TFIIF with a DNA containing the Ad-MLP also did not overcome the observed inhibition by the anti-RAP 30 antibodies (lanes 2 and 3). In order to further study if TFIIF was able to associate with the other factors so that it became resistant (or partially resistant) to inactivation by anti-RAP 30 antibodies, different combinations of factors were incubated in the absence of DNA prior to the addition of the antibodies. Incubation of TFIIF with TFIIE did not affect the inhibition of transcription by the anti-RAP 30 antibodies (Fig. 5B, lane 3). The incubation of TFIIF with RNA polymerase II and TFIIE (lanes 4 and 5), or in addition with TFIIEB (lanes 6 and 7) yielded low amounts of transcription resistant to the antibodies. This was increased approximately 3-fold when all the transcription factors were preincubated (lane 9); however, they were 2–3-fold lower than the control reaction (in the absence of anti-RAP 30 antibodies (lane 1) or with β-

galactosidase antibodies (lanes 2, 4, 6, and 8)). The most consistent interpretation of these results is that TFIIF can associate with the other transcription factors in a protein complex that is partially resistant to inactivation by the antibody. This partial resistance could be because of an instability of the complex. This protein complex became more stable if it was associated with a DNA molecule that contained the MLP.

CONCLUSIONS

The studies presented here indicate that RAP 30, a protein isolated using affinity chromatography on RNA polymerase II columns (13), is a functional component of TFIIF, a newly discovered general transcription factor. This conclusion was based on the following observations: (i) the TFIIF:RAP 30 ratio was constant during purification through five chromatographic steps (Fig. 3B); (ii) the incubation of TFIIF with monoaflinity-purified anti-RAP 30 antibodies inhibited transcription; (iii) a preparation containing TFIIF and TFIIE complemented the transcriptional activity of RAP 30-depleted extracts (9). It has previously been demonstrated that preparations of TFIIF (8) (containing TFIIF), but not TFIIB, were able to interact with purified RNA polymerase II. The studies presented here support this observation, namely that the preparation of TFIIF (containing TFIIF) was able to complement the transcriptional activity of an extract depleted of RAP 30-74 (13). Further investigations will determine whether purified TFIIF and/or purified TFIIF interacts
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with RNA polymerase II. The studies of Zheng et al. (12) have demonstrated that a 27,000-dalton protein isolated from HeLa cells co-sedimented with RNA polymerase II. Furthermore, Burton et al. (19) have shown that RAP 30 interacted with RAP 74 and that both RAP 30 and RAP 74 were required for transcription from the major late promoter. Thus, it is possible that TFIIF contains both RAP 30 and RAP 74 or that TFIIF interacts with the polymerase and that TFIIE is the equivalent to RAP 74 and interacts with TFIIF. This remains to be further investigated using purified fractions.

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