An N-terminally Fused Xenopus Transcription Factor IIIA Synthesized in Escherichia coli Is Biologically Active*

Ching-I Pao, Te-Chang Lee, You-Di Liao, and Cheng-Wen Wu‡

From the Department of Pharmaceutical Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794

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A 1.5-kilobase DNA fragment containing the Xenopus transcription factor IIIA (TFIIIA) gene was inserted into the prokaryotic expression vector pIN-III(A) containing the lpp/lac promoter. The recombinant DNA was introduced into Escherichia coli K-12 strain SB221. The expression TFIIIA gene was induced by isopropyl-β-D-thiogalactopyranoside, which resulted in the synthesis of a recombinant TFIIIA with an extra 17 amino acids fused to its N terminus as predicted from the nucleotide sequence. The engineered gene product, purified to at least 90% homogeneity, retained its binding affinity to the intragenic control region of the 5 S RNA gene, as well as its activity to stimulate 5 S RNA gene transcription in vitro.

The transcription of Xenopus 5 S RNA gene by RNA polymerase III requires at least three protein factors (TFIIIA, TFIIIB, and TFIIIC).1 Only TFIIIA has been purified to homogeneity from immature Xenopus oocytes where it is associated with 5 S RNA to form a 7 S particle (1–3). The amino acid sequence of TFIIIA has been deduced from a cDNA clone (4) and is in agreement with its amino acid composition published previously (3). This protein consists of 344 amino acids in a single polypeptide chain with a molecular weight of about 38,500 (3, 4).

TFIIIA is specifically required for the accurate initiation of 5 S RNA gene transcription by binding to an intragenic control region located at the center of the 5 S RNA gene. In the presence of TFIIIB and TFIIIC, a stable initiation complex is formed for multiple rounds of 5 S RNA synthesis (5). Deletion of the intragenic control region results in the concomitant loss of TFIIIA binding and transcriptional activities of the 5 S RNA gene (6, 7). In addition to the specific binding to the 5 S RNA gene required to initiate transcription, TFIIIA exhibits a high affinity for single-stranded DNA (8), promotes the reassociation of complementary single-stranded DNA into the double-stranded form (9), and has a DNA-activated ATPase activity (10).

Although TFIIIA has multiple functions, the structure/function relationships of TFIIIA are still unclear. With the development of recombinant DNA technology, the manipulation of the cloned gene on certain specific sites will provide the information necessary for the understanding of how TFIIIA is involved in the regulation of 5 S RNA gene transcription. In this paper, we describe the cloning of the TFIIIA gene into the bacterial expression vector pIN-III(A) (11), the expression of this cloned gene in Escherichia coli, and the purification and characterization of the engineered gene product. These are the necessary steps for further investigation of the structure and function of this important transcription factor.

MATERIALS AND METHODS

Plasmids and Bacteria Strain—Plasmid pIN-III(A), generously provided by Dr. Masayori Inouye, was used as the expression cloning vehicle (11). Plasmid puc3al.b, containing TFIIIA cDNA from Xenopus laevis, was a kind gift from Dr. R. G. Roeder (4). Plasmid pXba201 containing the 5 S RNA gene was used for transcription and footprinting analyses. The plasmid DNA was purified by a CaCl2-ethidium bromide sedimentation equilibrium method (12). E. coli K-12 strain SB221 (lpp lac F' hsdR302 16 lawmakers recA1 lacZ158 proA1 lacZ59) was used as a recipient for the constructed recombinant DNA.

Recombinant DNA Construction and Bacterial Transformation—A 1.5-kilobase fragment containing TFIIIA cDNA was obtained from plasmid puc3al.b after EcoRI digestion. This fragment was ligated with the vector pIN-III(A), which was linearized by EcoRI and diphosphorylated by calf intestine alkaline phosphatase (Boehringer Mannheim). The ligated product was then transformed into competent E. coli SB221 cells (13), and the transformed colonies were selected by agar plates containing 50 μg/ml ampicillin. The colonies were further screened for the correct orientation by restriction enzyme digestion analysis and for the TFIIIA gene expression by immunoprecipitation. The recombinant DNA with insert in the correct reading frame and the sense orientation with the lpp/lac promoter was designated as pIN-III(A)-FA. The constructed recombinant DNA is illustrated in Fig. 1.

Bacterial Culture and Induction of TFIIIA Synthesis—Five ml of overnight culture of E. coli strain SB221 containing plasmid pIN-III(A)-FA was inoculated to 500 ml of M9-glucose medium (14) supplemented with 20 μg/ml tryptophan, 0.5% casamino acids, and 50 μg/ml ampicillin in a 2-liter flask. The bacteria were grown at 37 °C until the optical density at 600 nm was 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG, Calbiochem) and ZnCl2 were added to the final concentrations of 1 mM and 10 μM, respectively. The incubation was continued for another 2 h at which point the cells were harvested by centrifugation at 5000 × g for 10 min. The pellets were washed once with 20 mM Tris (pH 8.0) containing 20 mM NaCl and 1 mM EDTA and stored at -70 °C. Approximately 3.5–4.0 g of cell pellet was obtained per liter of culture.

Purification of Recombinant TFIIIA—For a large preparation of recombinant TFIIIA, 20 g of cell pellet was thawed and suspended in 10 volumes (w/v) of lysing buffer which contained 50 mM Tris (pH 7.5), 200 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma) and then lysed by the addition of freshly prepared lysozyme (300 μg/ml, Sigma) at 4 °C for 1 h. After the addition of MgCl2 to a final concentration of 10 mM, the viscosity of the lysate was reduced by incubation with 10 μg/ml DNase I at 4 °C for 30 min. The crude lysate was centrifuged at 15,000 × g for 1 h. The supernatant was saved, and the pellet was resuspended in 100 ml of lysing buffer with gentle stirring for 30 min.
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at 4 °C. The debris was removed by centrifugation at 15,000 × g for 1 h. The two supernatants were combined and designated as fraction I. Fraction I was adjusted to 45% ammonium sulfate saturation and centrifuged at 10,000 × g for 20 min. The pellet was dissolved in 30 ml of 2 × buffer A (100 mM Tris, pH 7.5, 3 mM MgCl₂, 2 mM dithiothreitol, and 0.4 mM PMSF, containing 200 mM KC1 and dialyzed against the same buffer. The dialysate was designated as fraction II. An equal volume of 10 mM urea was added to fraction II, and the sample was applied to a Bio-Rex 70 (100-200 mesh, Bio-Rad) column (1.5 × 25 cm) which was pre-equilibrated with 300 ml of buffer A containing 100 mM KC1 and 5 mM urea. After washing the column with the same buffer, the proteins adsorbed to the column were eluted sequentially with 0.25, 0.5, and 1 M KC1 in buffer A containing 10 μM ZnCl₂. The fractions containing TFIIIA as determined by immunoprecipitation were pooled and designated as fraction III. The fraction III was dialyzed against buffer A containing 0.5 M KC1 and 10 μM ZnCl₂. The dialysate was adjusted to 85% ammonium sulfate saturation and then centrifuged at 10,000 × g for 20 min. The pellet was dissolved in 1 ml of column buffer and applied to a Sephacryl S-300 (Pharmacia LKB Biotechnology Inc.) column (1 × 120 cm) pre-equilibrated with 300 ml of 50 mM Tris buffer (pH 7.5) containing 2 mM NaCl, 5 mM MgCl₂, 2 mM dithiothreitol, 0.2 mM PMSF, and 10 μM ZnCl₂. Fractions containing recombinant TFIIIA as determined by in vitro transcription assay were pooled and designated as fraction IV. Fraction IV was dialyzed against buffer A containing 0.5 M KC1, 10 μM ZnCl₂, and 10% glycerol and then stored at −70 °C. Protein concentration was determined by the Bradford method (15) with bovine serum albumin as the standard.

In Vitro Transcription Assay—The Xenopus oocyte extract, prepared according to the method of Glick et al. (16), was fractionated into three fractions, A, B, and C, by phosphocellulose (Whatman, P-11) column chromatography (17). Fractions B and C provide RNA polymerase III and all the essential factors for 5 S RNA synthesis in vitro except TFIIIA. The native Xenopus TFIIIA was obtained by digesting the purified 7 S RNA fragment with RNase A as described by Hanas et al. (18). Briefly, a 305-bp DNA fragment containing the initiation codon of TFIIIA gene was obtained from plasmid pXbs 201 DNA, fraction B, fraction C, ATP, CTP, UTP, and [α-32P] GTP as described previously (19) in the presence of 10 units of RNasin (Promega). After incubation at 22 °C for 2 h, the reaction was stopped and the transcripts were purified by phenol extraction, precipitated by 70% ethanol, and analyzed by 7% polyacrylamide gel electrophoresis. After autoradiography, the band corresponding to 5 S RNA was excised, and the amount of radioactivity was determined by Cerenkov counting.

DNase I Protection Assay—The binding activity of recombinant TFIIIA to the intragenic control region of the 5 S RNA gene was monitored by the DNase I protection or footprinting technique as described by Hanas et al. (18). Briefly, a 305-bp DNA fragment containing the 5 S RNA gene was obtained from plasmid pXbs 201 by EcoRI and BamHI digestion. The DNA fragment was 32P-labeled at the 3′ end of the coding strand by filling the EcoRI restriction site with [γ-32P] ATP and [α-32P] GTP using avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories). After binding TFIIIA to the DNA fragment (0.5 nm) the complex was digested by DNase I (2 μg/ml) at room temperature for 1 min. The reaction was stopped by the buffer containing 10 mM Tris (pH 8.0), 40 mM EDTA, and 10 μg/ml sheared calf thymus DNA. The DNA precipitated by ethanol was electrophoresed on a 0.1% sodium dodecyl sulfate, 12.5% polyacrylamide gel and transferred to nitrocellulose paper. The conjugation of antisera against TFIIIA and the second antibody (goat anti-rabbit IgG conjugated with alkaline phosphatase, Kirkegaard & Perry Laboratories Inc.) to the blots was performed as described previously (20). The immunoblots were incubated in 50 mM sodium glycinate, pH 9.6, containing 0.5 mg/ml 5-bromo-3-chloro-4-indolyl phosphate, 0.1 mg/ml p-nitro blue tetrazolium chloride, 4 mM MgCl₂, and 0.5% Tween 20.

**RESULTS**

**Plasmid Construction**—The 1518-bp EcoRI fragment from plasmid puc3al.b contains the full length of TFIIIA cDNA with its own initiation codon (4). This fragment was inserted into the EcoRI site on the pIN-III(A) vector to construct the recombinant plasmid pIN-III(A)-FA (Fig. 1A). In the constructed recombinant plasmid pIN-III(A)-FA, the initiation codon of the TFIIIA gene is located 51 bp downstream from the initiation codon of the lipoprotein gene carried in the

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**FIG. 1. Construction of pIN-III(A)-FA.** A, the 1.5-kilobase EcoRI fragment containing TFIIIA gene was inserted into an expression vector pIN-III(A). The direction of transcription is indicated by an arrow. The lac I gene, the lpp promoter, the lac promoter/operator, and the β-lactamase gene are indicated by the hatched boxes. B, the nucleotide and the predicted amino acid sequences between the XbaI site on pIN-III(A) vector and the initiation codon of TFIIIA gene. The translation initiation site and Shine-Dalgarno sequence are shown in **bold type**.
pIN-III(A) vector (Fig. 1B). Therefore, the induced gene product could be a fused TFIIIA with an extra 17 amino acids on its N terminus, since the translation should start at the first initiation codon on the vector. The amino acid sequence of the N-terminal region predicted from the nucleotide sequence is also shown in Fig. 1B.

Expression of Recombinant TFIIIA—The expression of TFIIIA gene in the transformed E. coli could be detected by Western blotting (Fig. 2) and immunoprecipitation (data not shown). The protein band with molecular weight of about 41,000 was confirmed to be the recombinant TFIIIA, since it was detected only in the extract of E. coli transformed with plasmid pIN-III(A)-FA and induced by IPTG (Fig. 2, lane 1), but not in the extract of the similarly transformed cells without IPTG induction (Fig. 2, lane 2). Neither the extract of E. coli transformed with plasmid pIN-III(FA) but having the insert in the reverse orientation nor that transformed with the vector (pIN-III(A)) alone showed any activity in the immunoblotting assay (data not shown). The difference in molecular weights between native and recombinant TFIIIA is in agreement with the presence of the predicted 17 amino acids on the N terminus of the engineered gene product.

In addition to TFIIIA, two protein bands with molecular weights close to 32,000 and 52,000 were also observed on the immunoblot. The smaller protein species was probably a degradation product of the recombinant TFIIIA. The saturation of antiserum with Xenopus TFIIIA resulted in the loss of the antigenicity of this polypeptide as well as the putative recombinant TFIIIA; furthermore, it was not observed in the sample extracted from the pellet of cells without the IPTG induction (Fig. 2, lane 2). The larger protein species might be of bacterial origin, since it was observed in the sample extracted from E. coli both with or without the IPTG induction, and the antigenicity could not be deprived by the antiserum saturated with native Xenopus TFIIIA.

Fig. 2. The immunoblotting analysis of recombinant TFIIIA. Two hundred µg of protein in crude extract were electrophoresed on a 0.1% sodium dodecyl sulfate-12.5% polyacrylamide gel, transferred to nitrocellulose paper, conjugated with crude antiserum, and stained as described under “Materials and Methods.” Lane 1, crude extract of E. coli induced by IPTG; lane 2, crude extract of E. coli without the IPTG induction.

Purification of Recombinant TFIIIA—The immunoprecipitation and transcription assays were used to monitor TFIIIA activities in the development of a purification scheme. The results of purification of recombinant TFIIIA are summarized in Table I, and the protein components of samples obtained from various purification steps analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis are shown in Fig. 3. Ammonium sulfate at 45% saturation was able to remove 80–85% of excess proteins from the crude lysate (fraction I). The elution profile of the Bio-Rex 70 column is shown in Fig. 4. With 5 M urea in the column buffer, a great amount of nucleic acids was found in the flow through, as indicated by the absorption spectrum. Elution with buffer A containing 0.25 M KCl removed the urea remaining in the column and some of the loosely bound proteins, while 0.5 M KCl removed at least three major proteins having molecular weights smaller than TFIIIA. Finally, TFIIIA was eluted with buffer A containing 1.0 M KCl (fraction III). A considerable amount of TFIIIA was eluted if buffer A containing 0.5 M KCl instead of 0.25 M KCl was used as the first eluent, presumably due to

![Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the samples obtained at various purification steps.](image)

**Table I**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification factor</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>646</td>
<td>1.6 x 10⁶</td>
<td>3.9 x 10⁵</td>
<td>-fold</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>92</td>
<td>1.6 x 10⁴</td>
<td>1.7 x 10³</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>III</td>
<td>3.2</td>
<td>1.4 x 10⁵</td>
<td>4.4 x 10³</td>
<td>4</td>
<td>87</td>
</tr>
<tr>
<td>IV</td>
<td>0.16</td>
<td>3.2 x 10⁴</td>
<td>2.0 x 10²</td>
<td>118</td>
<td>20</td>
</tr>
</tbody>
</table>

Twenty grams of cell pellet were lysed, and the recombinant TFIIIA was purified as described under “Materials and Methods.” Aliquots of fractions I, II, III, and IV were used to determine the total protein content and the transcriptional activities. Since the accurate determination of transcriptional activity could not be made with the crude extract (fraction I), the 45% ammonium sulfate precipitate (fraction II) was assumed to have 100% yield.

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the samples obtained at various purification steps. Samples were electrophoresed on a 0.1% sodium dodecyl sulfate-12.5% polyacrylamide gel at 150 V for 1 h using a discontinuous pH system of Laemmli (25). After electrophoresis, the gel was stained with 0.2% Coomassie Brilliant Blue R (Sigma) in 40% methanol and 10% glacial acetic acid, and destained in 40% methanol and 10% acetic acid. Lane 1, fraction I, crude extract (50 µg); lane 2, fraction II, ammonium sulfate precipitate (40 µg); lane 3, fraction III, 1 M KCl eluate of the Bio-Rex 70 column (24 µg); lane 4, fraction IV from the Sephacryl S-300 column (6 µg); lane 5, purified Xenopus TFIIIA (2 µg).
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Transcription Factor IIIA

Synthesized from E. coli

FIG. 4. Elution profile of the Bio-Rex 70 column chromatography. Sample (fraction II) in buffer A containing 5 M urea and 100 mM KCl was applied to the Bio-Rex 70 column (1.5 × 20 cm), and proteins adsorbed on the column were sequentially eluted with 0.25, 0.5, and 1 M KCl in buffer A containing 10 μM ZnCl₂. The flow rate was 0.8 ml/min, and fractions of 5 ml were collected.

FIG. 5. Elution profile of the Sephacryl S-300 gel filtration column chromatography. Sample (fraction III) was concentrated and applied to the Sephacryl S-300 column (1 × 120 cm) pre-equilibrated with 50 mM Tris (pH 7.5) containing 2 M NaCl, 5 mM MgCl₂, 2 mM dithiothreitol, 0.2 mM PMSF, and 10 μM ZnCl₂. Fractions of 1 ml were collected at a flow rate of 5 ml/h.

The effect of residual urea remaining in the column. As shown in Table I, approximately 95% of the total protein in fraction II could be removed by the Bio-Rex 70 column chromatography, and thus the purity of recombinant TFIIIA was increased up to 40–45% by this step as determined by densitometry of the stained gel (Fig. 3).

The elution profile of the Sephacryl S-300 column is shown in Fig. 5. As can be seen in the figure, there are three peaks in the elution profile, and the TFIIIA activity was found in the first peak. Fractions containing the highest activity (fractions 53–56) were pooled and designated as fraction IV. The recombinant TFIIIA in fraction IV was at least 90% pure, as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). The final yield of recombinant TFIIIA in fraction IV was about 20% (Table I).

DNA Binding and Transcription Activities of Recombinant TFIIIA—The DNase I protection patterns showing the binding of native Xenopus TFIIIA (Panel A) and the recombinant TFIIIA (Panel B) to the 5 S RNA gene are presented in Fig. 6. The protection patterns of the intragenic control region produced by native TFIIIA and the recombinant TFIIIA are essentially identical. The protein concentration required for the full protection of the intragenic control region is about 12.5 nM for the recombinant TFIIIA, which is 2–3-fold higher than that for the native Xenopus TFIIIA (5 nM) obtained from the 7 S particle by RNase A digestion. However, the amounts of 5 S RNA synthesis stimulated by the recombinant and native TFIIIA are almost the same as the protein concentration varying from 2 to 32 nM (Fig. 7). Thus, it appears that the recombinant TFIIIA, in spite of the extra 17 amino acids present at its N terminus, shows similar activities in binding to the 5 S RNA gene and in promoting the 5 S RNA gene transcription.

DISCUSSION

The experimental results presented herein indicate that the Xenopus TFIIIA gene was expressed in E. coli by using the prokaryotic expression vector pIN-III(A). The TFIIIA cDNA was inserted into the lipoprotein gene on the vector, which was controlled under lpp/lac promoter, and, therefore, a N-terminally fused TFIIIA was produced. In order to obtain a nonfusion TFIIIA, a 27-bp fragment located between the XbaI and EcoRI restriction sites was deleted to remove the initia-
The structure of TFIIIA can be roughly divided into two parts: the 9 loop-like domains extending from residues 13 to 276 and the remaining approximately 70 amino acids at the C terminus (22, 24). The nine repeating units bind to the 3′-half of the intragenic control region of the 5 S RNA gene, whereas the C-terminal region presumably interacts with other transcription factors or RNA polymerase III (22). From the results of the transcription and DNase I protection assays, it was found that the activity of recombinant TFIIIA is comparable with that of the native Xenopus TFIIIA. These results imply that the presence of an extra 17 amino acids at the N terminus of TFIIIA does not particularly affect the structure so as to alter its biological functions. They also suggest that the N-terminal region of TFIIIA, which binds tightly to the 3′-half of the intragenic control region, is not involved in positioning the transcriptional machinery for proper initiation. Thus, a certain degree of topological freedom seems to be allowed in the formation of transcription initiation complex.

This is the first report to describe the expression of an eukaryotic transcription factor of RNA polymerase III in E. coli and the detailed purification method of the engineered gene product. The system developed here may be used as a tool to elucidate the structure/function relationships of TFIIIA, particularly in light of the multiple functions this protein appears to have. including binding to 5 S DNA and RNA, ATP hydrolysis, DNA reassociation, as well as formation of the initiation complex during the 5 S RNA gene transcription.

REFERENCES

FIG. 7. In vitro transcription of 5 S RNA gene stimulated by native and recombinant TFIIIA. Assays were performed as described under “Materials and Methods.” Either native Xenopus TFIIIA obtained from 7 S particle by RNase A digestion (panel A) or recombinant TFIIIA from fraction IV (panel B) was added into the reaction mixture. The final protein concentrations used were: lane 1, 2 nM; lane 2, 4 nM; lane 3, 8 nM; lane 4, 16 nM; lane 5, 32 nM for both native and recombinant TFIIIA.