High yield purification of active transcription factor IIIA expressed in E.coli

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ABSTRACT
Transcription factor IIIA (TFIIIA), a sequence-specific DNA-binding protein from Xenopus laevis, is a zinc finger protein required for transcription of 5S rRNA genes by RNA polymerase III. We describe the purification and characterization of recombinant TFIIIA (recTFIIIA) expressed in E. coli. RecTFIIIA was purified to greater than 95% homogeneity at a yield of 2–3 milligrams per liter of bacterial culture. This purified protein protects the internal control region of a 5S rRNA gene from DNase I digestion, yielding footprints on both strands identical to those produced by the ovarian protein (ovaTFIIIA). Quantitative analysis of binding data from gel retardation assays yielded a Kᵩ of about 0.4 nM for TFIIIA from either source. Using a quantitative TFIIIA-dependent in vitro transcription assay, we found that recTFIIIA is equivalent to ovaTFIIIA in supporting transcription of 5S rRNA genes. We conclude that recTFIIIA is functionally indistinguishable from the protein purified from Xenopus ovaries, and can be readily obtained in pure form and large quantity.

INTRODUCTION
Transcription Factor IIIA (TFIIIA) is a sequence-specific DNA-binding protein required for transcription initiation by RNA polymerase III on 5S rRNA genes (1–3). TFIIIA binds specifically to the internal control region of the 5S rRNA gene, protecting nucleotides +45 to +96 from DNase I digestion (1,4,5). Although two or more other factors are required for efficient transcription of 5S rRNA genes (2,3,6–8), TFIIIA is likely to be the primary protein acting to establish sequence specificity in transcription initiation. TFIIIA is a zinc metalloprotein (9), containing 344 amino acids (10) and as many as 9 Zn²⁺ ions (11). In fact, it is the prototype ‘zinc finger’ protein, containing nine tandemly repeated zinc finger motifs (11). In addition to its DNA-binding and transcription factor activities, TFIIIA also acts as an RNA-binding protein, associating with 5S RNA to form a 7S ribonucleoprotein particle (12), believed to function as a storage particle for 5S rRNA in immature oocytes of Xenopus (13).

Because of its key role in 5S rRNA gene transcription, its status as the first Zn²⁺ finger protein identified, and its unusual function as both a sequence-specific DNA and RNA-binding protein, detailed analysis of the structure and function of TFIIIA is of considerable interest. Availability of large quantities of purified protein for biophysical studies and the ability to produce mutant forms of TFIIIA will be necessary for structure-function analyses. Previously described methods for expression of TFIIIA in E. coli result in the production of rather small quantities of the protein, fail to yield protein of high purity, or produce a polypeptide containing additional foreign amino acids at the N-terminus (14–17). While these methods may yield material suitable for some analyses, published procedures are not readily adaptable for the production of wild-type or mutant forms of TFIIIA that can be used in quantitative biochemical and/or biophysical studies.

We describe here the application of the in vivo T7 RNA polymerase expression system developed by Studier and colleagues (18) to the production and purification of functional TFIIIA in E. coli. We obtain 2–3 mg of TFIIIA at greater than 95% purity from 1 liter of bacterial culture, or even higher quantities with a small sacrifice in purity. This purified protein is functionally indistinguishable from TFIIIA purified from Xenopus ovaries.

MATERIALS AND METHODS
Bacterial Strains and Vectors
pGA11 (19) is a phagemid derived from the vector pGP12 (19) and contains the EcoRI fragment of the X. laevis TFIIIA cDNA clone pUC3al.b of Ginsberg et al. (20) cloned into the unique EcoRI site of pGP11 in the orientation permitting rescue of phage containing the sense strand of TFIIIA DNA. Using site-directed mutagenesis (19) with an oligonucleotide of sequence 5’-TCT-CTCCCCATATGTCCTTCAGCA-3’, we altered the sequence at and around the initiator methionine codon of TFIIIA to include an NdeI site. This plasmid was designated pGA11-NdeI. The NdeI/BamHI fragment of pGA11-NdeI containing the TFIIIA sequence was cloned into pET-llb (18), similarly digested with NdeI and BamHI, to obtain pTA101. In separate experiments, the X. laevis TFIIIA coding sequence in phagemid pGA1 (19)
at codons 300–304 was changed to 5′-GGT TAC ATC CCG CCG-3′ using oligonucleotide-directed mutagenesis as previously described (19). The sequence of the oligonucleotide used was 5′-CTTTGCTTTCGGGGATGTAACCAGTGAGGC-3′. The new sequence in this region conserves the amino acid sequence of TFIIIA, but changes the codons used for residues 300 and 302–304 from rare usage codons to high usage codons in E. coli (20,21). We have called the resulting phagemid pGA1:EP(300–304). From pGA1:EP(300–304), we isolated an XhoI/BamHI fragment containing the 3′ end of the TFIIIA cDNA sequence, including the sequence changes at codons 300–304. This fragment was exchanged for the corresponding XhoI/BamHI fragment in pTA101 to generate pTA102 (figure 1).

p9Xbs201 contains the X. borealis somatic-type SS rRNA gene from the EcoRI/BamHI fragment of plasmid pXbs201 (22) cloned into pUC9 similarly digested with EcoRI and BamHI. Prior to digestion with EcoRI and BamHI, the unique HindIII site of pUC9 had been removed by digestion, fill-in and religation. pST5RD-Xhol was derived from pST5RD (19) by introducing an Xhol site immediately downstream of the SS rRNA gene termination signal using the oligonucleotide 5′-GGGTTTTGCTCGAGTGCCATTCTG-3′ in a site-directed mutagenesis experiment. Digestion of pST5RD-Xhol with Xhol produces a 199-bp DNA fragment containing the entire SS rRNA gene from −49 to +124 with some flanking poly linker sequence at the 5′ end.

In general, E. coli K-12 strain NM522 (23) was used for growth, isolation, and analysis of the DNA vectors. E. coli B strain BL21(DE3) (F−ompT r−) (18,24) was used to express the cloned TFIIIA gene.

SDS-Polyacrylamide Gel Electrophoresis

To assess the composition and purity of various fractions, proteins were separated on 12% polyacrylamide SDS gels as described (25).

Western Blot Analysis

Transfer of proteins from SDS polyacrylamide gels was performed with a Bio-Rad Trans-blot cell to 0.45 μM nitrocellulose (BioTrace-NT, Gelman Sciences) in 0.096 M glycine/0.0125 M Tris base at 70 volts for 3 hours at 4°C. Following transfer, the filter was preblocked in 5% (w/v) nonfat dry milk/0.02% NaN₃/0.096 M HEPES (N'-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), pH 7.4/140 mM NaCl/0.1% Tween 20 (polyoxyethylene sorbitan monolaurate)) for 1–3 hours at room temperature with gentle shaking. The blot was washed two times with TBS/Tween and then incubated with rabbit anti-serum raised against purified ovarian TFIIIA in 50 ml TBS/Tween for 2 hours at 37°C with gentle rocking. Unreacted antibody was removed by washing two times in TBS/Tween, one time in HSTBS/Tween (10 mM Tris-HCl, pH 7.4/1.0 M NaCl/0.5% Tween 20), and three times in TBS/Tween. The blot was then incubated with 125I-protein A (ICN) (10–15 μCi) in 50 ml TBS/Tween for 2 hours at 37°C with gentle rocking. Excess 125I-protein A was removed by washing two times in TBS/Tween, one time in HSTBS/Tween, and three times in TBS/Tween. The blot was dried and exposed to Kodak XAR film at −70°C.

Ovarian TFIIIA Purification

TFIIIA was purified from immature X. laevis ovaries as described by Smith et al. (26) and as modified by Campbell and Setzer (27).

Recombinant TFIIIA Purification

A liter of rich medium (25 g Bactotryptone/15 g yeast extract/5 g NaCl/pH 7.0) containing 100 μg/ml ampicillin and 4 g/liter glucose was inoculated with 9 ml of an overnight culture of BL21(DE3)/pTA102. The culture was grown at 37°C with shaking at 250 rpm to an OD₆₀₀ of 0.4 to 0.6, and then supplemented with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37°C for 3–4 hrs. Cells were harvested by centrifugation at 6,000 × g in a Sorvall GS3 or GSA rotor and were washed once with 15 ml buffer A (20 mM NaH₂PO₄/100 mM MgCl₂/40 mM DTT (dithiothreitol)/50 μM ZnSO₄/10% glycerol) containing 250 mM NaCl. The washed cellular pellet was resuspended in 15 ml buffer B/250 mM NaCl/1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated for a total of 2–6 minutes with frequent cooling in ice. The sonicated extract was spun for 10 minutes at 6,000 × g in a SS-34 rotor, and the resulting pellet resuspended in 15 ml buffer C/250 mM NaCl/10 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated for a total of 2 minutes with frequent cooling in ice. An additional 15 ml of buffer D was added to the 250 mM NaCl/10 mM phenylmethylsulfonyl fluoride. The resuspended pellet was sonicated again for a total of 2 minutes with frequent cooling on ice. The supernatant was added to 20 minutes at 11,000 × g. The supernatant (crude TFIIIA extract) was carefully discarded. We refer to this fraction as S2.

Buffer A/250 mM NaCl/5 M urea saturated with (NH₄)₂SO₄ at 4°C was added to the S2 fraction to bring it to 40% saturation with (NH₄)₂SO₄ and mixed at 4°C for 1 hour. The 40% (NH₄)₂SO₄ precipitate was pelleted by centrifuging for 20 minutes at 11,000 × g. Additional buffer A/250 mM NaCl/5 M urea saturated with (NH₄)₂SO₄ was added to the 40% (NH₄)₂SO₄ supernatant to achieve 80% (NH₄)₂SO₄ saturation
and mixed at 4°C for 1 hour. After the sample was centrifuged for 20 minutes at 11,000 × g, the 80% (NH₄)₂SO₄ supernatant was decanted and the pellet was redissolved in buffer A/5 M urea, using 3.4 ml of buffer per 0.1 ml of pellet.

The 40–80% (NH₄)₂SO₄ fraction was applied to a 3 ml BioRex 70 column (BioRad) pre-equilibrated in buffer A/250 mM NaCl/5 M urea. After washing the column with the same buffer, bound protein was eluted with a linear 18 ml gradient of NaCl (250 mM to 1 M in buffer A/5 M urea). Following analysis of column fractions on silver-stained SDS-polyacrylamide gels (28), fractions enriched for TFIIIA were pooled and dialyzed (Spectra/Por molecularporous membrane #3) overnight against buffer A/1.2 M (NH₄)₂SO₄.

Following dialysis, the pooled BioRex 70 fractions were filtered through a 0.45 micron Millex-HV filter (Millipore) to remove insoluble material. The resulting solution was applied to a 1 ml phenyl-Superose column (Pharmacia) pre-equilibrated with buffer A/1.2 M (NH₄)₂SO₄. Proteins were eluted with a reverse linear 18 ml gradient of (NH₄)₂SO₄ (1.2 M to 0.5 M in buffer A), followed by a step gradient (0.5 M to 0 M (NH₄)₂SO₄). Column fractions were analyzed on silver-stained SDS-polyacrylamide gels to identify fractions containing TFIIIA substantially free of other contaminants. These fractions were used as the source of recombinant TFIIIA in functional assays.

Protein Concentration Determination
The method of Bradford (29) was used to determine the concentration of TFIIIA in the recombinant and ovarian preparations. Bovine serum albumin (BSA) was used as the standard, and the final TFIIIA concentration was determined by multiplying the apparent concentration by 0.62 to account for differential binding of dye by TFIIIA and BSA (30).

Gel Retardation Assay
A 199-bp DNA fragment (Xhol fragment of pST5RD-Xhol) containing the X. borealis somatic-type 5S rRNA gene was labeled using [α-³²P]dCTP and the Klenow fragment of E. coli DNA polymerase I. TFIIIA was incubated at a concentration of 8 nM with variable concentrations (1–9.1 nM) of the labeled fragment in 20 μl reactions containing buffer B (20 mM Tris-Cl, pH 7.5/7 mM MgCl₂/10 μM ZnCl₂/1 mM DTT/10% glycerol/70 mM KCl) supplemented with 10 μg/ml poly(dI-dC) and 100 μg/ml BSA. After incubating the reactions at 25°C for 30 minutes the samples were quickly cooled on ice and then immediately loaded onto a non-denaturing 6% (0.12% bisacrylamide) polyacrylamide gel containing 5% glycerol. The gel was precooled to 4°C and pre-run at 300 volts for 30–60 minutes. The gel and running buffer consisted of 0.025 M Tris base/0.2 M glycine. Electrophoresis was for 3 hours at 4°C. Wet gels were dried on cellophane (Bio-Rad) prior to autoradiography. Cerenkov radiation in gel slices, corresponding to the ‘free’ and ‘bound’ bands on the autoradiographs, was measured (Beckman, LS 1801) to determine the amount of DNA which was free or complexed with TFIIIA. Scatchard analysis (31) of these data was used to estimate equilibrium dissociation constants (Kᵦₛ’s) and the concentration of active TFIIIA in our preparation, assuming a binding stoichiometry of 1:1 (26,32).

DNase I Protection
100 nanograms of DNA template (pC9Xbs201) containing the 5S RNA gene was labeled on the 5’-end of the noncoding strand (HindIII site) using [γ-³²P]ATP and T4 polynucleotide kinase after having dephosphorylated the 5’ ends with calf intestine alkaline phosphatase. Alternatively, the DNA was labeled on the 3’-end of the coding strand (HindIII site) using [α-³²P]dCTP and modified T7 DNA polymerase (33,34) (Sequenase, United States Biochemical). Secondary digestion with EcoRI removed one of the labeled ends onto a small DNA fragment that did not interfere with the subsequent footprints. The labeled DNA (1 nM) was preincubated at 25°C for 30 minutes with increasing amounts TFIIIA (1 nM, 5 nM, 10 nM and 20 nM) in buffer B supplemented with 100 μg/ml BSA. DNase I (18 ng) was added and the reactions incubated at room temperature for 90 seconds. The reactions were stopped by the addition of an equal volume of 150 mM NaCl/50 mM Tris-Cl, pH 8.0/5 mM EDTA, pH 8.0/0.5% SDS/50 μg/ml glycogen (SETS/glycogen). Following extraction with phenol and precipitation in ethanol, products were run on 8% polyacrylamide/8 M urea sequencing gels. Wet gels were transferred to Whatman 3M paper prior to autoradiography.

Transcription Assay
A TFIIIA-dependent in vitro transcription assay was used to determine the transcriptional activity of the recombinant TFIIIA. Template DNA (pC9Xbs201) (2.5 nM) was preincubated at 25°C for 60 minutes in a 20 μl reaction containing buffer B, 5 μl of a Dignam nuclear extract (35) prepared from a X. laevis kidney cell line, 20 units RNAsin (Promega), and variable concentrations of TFIIIA (0 nM, 0.05 nM, 0.2 nM, 0.5 nM, 0.8 nM, 1.5 nM, and 3.0 nM). Following the preincubation period, 10 μCi [α-³²P]UTP (800 Ci/mmole) was added along with ATP, GTP, and CTP to a final concentration of 500 μM and UTP to a final concentration of 50 μM. Transcription proceeded for an additional 60 minutes, and was then stopped by the addition of 150 μl of SETS/glycogen. Following extraction with phenol and precipitation in ethanol, transcription products were resolved on a 10% polyacrylamide/8.33 M urea gel. Wet gels were dried...
### RESULTS

#### Expression and Purification of TFIIIA

We have made use of the lac-controlled, plasmid-based T7 RNA polymerase expression system of Studier and colleagues (18) for production of *Xenopus* TFIIIA in *E. coli*. This system is far superior to several others we or others (14–17) have tested, producing approximately 5–10 fold more TFIIIA in crude form than we have obtained using any other expression method. Following IPTG induction for 4 hours, TFIIIA constitutes 15%–20% of the total cellular protein (See figure 2 and Table I); this corresponds to about 60 to 70 milligrams of recTFIIIA per liter of culture. Based on sedimentation analysis, however, the recTFIIIA is in a highly aggregated and largely insoluble form in the initial crude extract (data not shown). We found that induction of cultures at 19°–25°C rather than at 37°C results in much higher solubility of the recTFIIIA; unfortunately, analysis of this protein by sedimentation on glycerol gradients suggests that, although soluble, the recTFIIIA remains aggregated in particles exhibiting a heterogeneous size distribution (data not shown). We also found that the aggregated and insoluble TFIIIA produced at 37°C can be partially solubilized by treatment with 5 M urea. These solubilized molecules are reduced to monomer form. Since yields of recTFIIIA are much higher at 37°C (data not shown), since 5 M urea treatment is known not to adversely affect activity of TFIIIA obtained from ovariates (26), and since protein produced at the lower temperatures of induction would nonetheless require urea treatment to promote disaggregation, we have elected to induce TFIIIA production at 37°C and solubilize, using 5 M urea, the initially highly aggregated protein found in the insoluble cell fraction following sonication and centrifugation. Subsequent purification has been carried out on this material. Approximately 60% of the recTFIIIA is solubilized in the initial 5 M urea extraction step, and recTFIIIA comprises about 25% of the protein in the resulting fraction. Overall, the 5 M urea treatment yields a 1.5-fold enrichment of recTFIIIA as compared to an untreated whole cell extract.

#### Purification of Recombinant TFIIIA from *Escherichia coli*

<table>
<thead>
<tr>
<th>Step</th>
<th>Values represent yields from 1 liter of culture</th>
<th>Step yield</th>
<th>Overall yield</th>
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<tr>
<td>Whole cell extract</td>
<td>% mg</td>
<td>%</td>
<td>%</td>
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<tr>
<td>S2 fraction</td>
<td>19 67</td>
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<td>58</td>
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<tr>
<td>40–80% (NH₄)₂SO₄ cut</td>
<td>42 30</td>
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<td>45</td>
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<tr>
<td>BioRex 70 pooled fraction</td>
<td>76 11.4</td>
<td>38</td>
<td>17</td>
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<tr>
<td>Phenyl-Superose fraction</td>
<td>&gt;95 2.4</td>
<td>21</td>
<td>4</td>
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a Protein purity determined from densitometric scanning of the SDS-polyacrylamide gel of figure 2.

b Concentration of TFIIIA calculated as follows:

\[
\text{Concentration of TFIIIA} = \left( \frac{\text{Sample's protein concentration} \times \text{Sample volume} \times (\text{% purity})}{0.62} \right)
\]

The method of Bradford (29) was used to determine the protein concentration of the samples. BSA was used as the standard, and the final concentration of TFIIIA was determined by multiplying the apparent concentration by 0.62 to account for differential binding of dye by TFIIIA and BSA (30).

c Estimated from the SDS-polyacrylamide gel in figure 2 where the TFIIIA in the phenyl-Superose fraction (lane 1) was used as a standard.

We tested the solubility of TFIIIA relative to other polypeptides in the 5 M urea extract at a variety of (NH₄)₂SO₄ concentrations. Using a 40%–80% (NH₄)₂SO₄ cut, we can achieve an additional 1.5 fold enrichment of recTFIIIA. In most cases (Table I, for example), the yield of recTFIIIA is quite high (greater than 75%). We have found some variability in recoveries at this step, however, and it can probably be eliminated with little or no effect on the ultimate purity of the final recTFIIIA preparation (data not shown). This may be advisable if high yield is particularly important.

We analyzed the behavior of recTFIIIA on a variety of ion exchange and affinity columns, and found that substantial and roughly equivalent purification can be achieved on a number of resins, including hydroxyapatite, phosphocellulose, BioRex 70, and MonoS (data not shown). We detected no binding, and minimal purification, on a MonoQ column under the conditions tested. For our purposes, we elected to further purify the 40%–80% (NH₄)₂SO₄ fraction by chromatography on BioRex 70 in the presence of 5 M urea. Using a 250 mM to 1 M linear NaCl gradient, recTFIIIA elutes from the column with a peak at about 400 mM NaCl. Total recovery from the column is high, and recovery in the pooled fractions may be as high as 40–60%.

### Table I. Purification of Recombinant TFIIIA from *Escherichia coli*

<table>
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Figure 5. DNase I protection assay. 1 nM end-labeled DNA (pC9Xbs201) was incubated with increasing amounts of recTFIIIA or ovaTFIIIA prior to digestion with DNase I. A. Non-coding strand footprint, lane 1: no DNase I added; lanes 2–10: 18 ng DNase I added for 90 seconds; lanes 3–6: 1 nM, 5 nM, 10 nM, or 20 nM recTFIIIA added, respectively; lanes 7–10: 1 nM, 5 nM, 10 nM, or 20 nM ovaTFIIIA added, respectively. B. Coding strand footprint, lane 1: no DNase I added; lanes 2–10: 18 ng DNase I added for 90 seconds; lanes 3–6: 1 nM, 5 nM, 10 nM, or 20 nM recTFIIIA added, respectively; lanes 7–10: 1 nM, 5 nM, 10 nM, or 20 nM ovaTFIIIA added, respectively.

Figure 4. TFIIIA binding to the 5S rRNA gene. A. Autoradiograph from a gel retardation experiment. Lane 1: no TFIIIA; lane 2: 6 nM recTFIIIA; lane 3: 6 nM ovaTFIIIA. The binding reaction in each case contained 8 nM 5S DNA (XhoI fragment from pST5RD-XhoI). ‘Bound’ indicates the position of the TFIIIA/DNA complex, and ‘Free’ indicates the position of the free DNA. B. Scatchard analysis of gel retardation assays performed with fixed amounts of recTFIIIA (*) or ovaTFIIIA (•) and variable amounts of 5S DNA. Binding reactions contained 8 nM TFIIIA and 1–9 nM DNA (XhoI fragment from pST5RD-XhoI). Data were fit to a straight line using a linear least squares analysis. The calculated equilibrium dissociation constants (K_D, equal to (−slope⁻¹)) are 0.43 ± 0.06 nM and 0.41 ± 0.03 nM for recTFIIIA and ovaTFIIIA, respectively. Errors given are derived from the standard error of the regression coefficient (K_A).

Table I. The major contaminant following BioRex 70 chromatography is a polypeptide of about 37,000 daltons that co-chromatographs with recTFIIIA on a variety of columns and reacts with mono-specific TFIIIA antibodies raised against purified ovarian TFIIIA (figure 3A). We therefore believe that this polypeptide is a truncated form of TFIIIA. Furthermore, the absence of an analogous shorter form of TFIIIA in preparations of a mutant form of protein lacking C-terminal sequences suggests to us that this shorter polypeptide may be truncated at the C-terminus (data not shown).

Prior to chromatography on phenyl-Superose, urea was removed from the TFIIIA preparation by dialysis while simultaneously introducing a high concentration of salt. The high salt concentration is necessary to ensure binding to phenyl-Superose and may also be necessary to guard against aggregation of the protein, since it exhibits limited solubility in the absence of urea and in the presence of low concentrations of salt. We have found considerable variation in the amount of recTFIIIA that precipitates and is lost during this dialysis step. In the example of Table I, recTFIIIA recovery following dialysis and filtration was only 60%, but yields are generally somewhat higher than this. We found that phenyl-Superose chromatography is necessary to separate full length recTFIIIA from the 37,000 dalton contaminant. RecTFIIIA elutes in a reverse linear salt gradient with a peak at about 0.95 M (NH_4)_2SO_4, while the 37,000 dalton truncated form elutes with a peak at about 0.86 M (NH_4)_2SO_4. Only the fractions at the leading edge of the recTFIIIA peak were pooled in order to minimize contamination with the 37,000 dalton polypeptide. These initial column fractions yielded 2–3 milligrams recTFIIIA at greater than 95% purity, based on densitometric scanning of coomassie blue stained SDS-polyacrylamide gels. Again, the total yield of recTFIIIA from the column is high, but only about 20–30% of the recTFIIIA is recovered free of the contaminating 37,000 dalton polypeptide (figure 3A). In some cases, we have pooled the remaining TFIIIA-containing fractions, supplemented the pooled fractions with additional salt, and re-chromatographed the pooled fractions on phenyl-Superose to obtain additional protein free of the 37,000 dalton contaminant. Such a procedure was not used to derive the data of figure 2.

These results are summarized in figures 2 and 3 and Table I. RecTFIIIA can be obtained at greater than 95% purity at a
yield of 2 – 3 mg per liter of bacterial culture, representing an overall yield of about 4%. On high resolution SDS polyacrylamide gels, the mobility of recTFIIIA is slightly greater than that of the ovaTFIIIA control (figure 3B), a property we have noted for TFIIIA expressed in E. coli using other vectors as well (data not shown). We have not determined the reason for this slight difference in electrophoretic mobility, but it does not appear to affect TFIIIA function, as we show below.

Functional Assays
We tested the activity of recTFIIIA relative to that isolated from immature oocytes of X. laevis (26) (ovaTFIIIA) using a variety of in vitro assays. We measured the equilibrium dissociation constants (KD) for binding of the two preparations of TFIIIA to a 199-bp DNA fragment containing the X. borealis somatic-type 5S rRNA gene. This was accomplished using a gel retardation assay to separate DNA fragments bound to TFIIIA from those that are unbound and analyzing the resulting data according to Scatchard (31). The data of figure 4B demonstrate that recTFIIIA and ovaTFIIIA bind to the 5S rRNA gene-containing DNA fragment with KD values of 0.43 ± 0.06 nM and 0.41 ± 0.03 nM, respectively. These errors reflect the precision obtained within a single experiment leading to the generation of one Scatchard curve. We have found that there is considerably greater variability in the KD values obtained in separate experiments; nonetheless, estimates of the KD generally vary by no more than a factor of two. The mobility of the recTFIIIA/DNA complexes in non-denaturing polyacrylamide gels is slightly greater than that of the ovaTFIIIA/DNA complexes (figure 4A), paralleling the difference in mobility seen with the purified proteins in SDS-PAGE analysis. Based on the data from the Scatchard curves, we estimate the recTFIIIA and ovaTFIIIA preparations to be 56% and 52% active, respectively, assuming a binding stoichiometry of 1:1 (26,32). Of course, these values are subject to both experimental and systematic errors in our determination of TFIIIA concentration, and may be underestimates.

Figure 6. TFIIIA-dependent in vitro transcription assay. As described in the text, 2.5 nM DNA (pC9Xbs201) was incubated with variable amounts (0 nM, 0.05 nM, 0.2 nM, 0.5 nM, 0.8 nM, 1.5 nM, 3.0 nM) of recTFIIIA (*) or ovaTFIIIA (□) and a fixed amount of a TFIIIA-deficient nuclear extract from a X. laevis kidney cell line prepared to permit the synthesis of 32P-labeled 5S RNA. Transcription products were separated on a denaturing polyacrylamide gel, cut out following autoradiography, and Cerenkov radiation measured to determine the level of transcriptional activation by the added TFIIIA.

DISCUSSION
Detailed structure-function studies of TFIIIA are of broad interest because of the unusual biochemical properties of the protein and the occurrence of TFIIIA-like structural features in a wide variety of nucleic acid-binding proteins. Unfortunately, such studies have been hampered by the lack of a convenient method for producing large amounts of pure wild-type and mutant forms of TFIIIA. Previous attempts to express Xenopus TFIIIA in E. coli using various methods (14 – 17) resulted in rather low yields of protein, relatively impure preparations, and/or proteins with foreign amino acids at the N-terminus. Precise quantitative measures of TFIIIA activity, which may prove to be particularly important in assessing the effects of various mutations on TFIIIA function, require pure preparations of protein. Biophysical studies of TFIIIA will require large quantities of the protein that are difficult to obtain directly from Xenopus and will benefit greatly from the availability of large quantities of TFIIIA mutants as well as wild-type protein. The method of production and purification we have described here will make these approaches feasible. We have shown that TFIIIA produced in and purified from E. coli is functionally indistinguishable, both qualitatively and quantitatively, from the protein isolated from Xenopus oocytes. It should therefore be possible to measure even small effects of specific mutations on TFIIIA function, as we show below.
protein function and to use recTFIIA to obtain structural information which will advance our understanding of Zn$^{2+}$ finger proteins in general.

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