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QUARTERLY

Maize TF IIIA — the first transcription factor IIIA from monocotyledons. Purification and properties*

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Purification and properties of transcription factor IIIA (TF IIIA) from maize pollen (*Zea mays* L.) are presented for the first time. The purified protein has a molecular mass of about 35 kDa and exibits binding affinity toward both 5S rRNA and 5S rRNA gene. It also facilitates transcription of the 5S rRNA gene in a HeLa cell extract.

Eukaryotic transcription factor IIIA (TF IIIA) is known to bind and specifically activate transcription of homologous 5S rRNA genes. The same protein also forms a 7S complex with mature 5S rRNA. Transcription of the ribosomal 5S RNA genes by eukaryotic RNA polymerase III in the nucleus depends on the transcription factors IIIA, IIIB and IIIC (TF IIIA, TF IIIB and TF IIIC). The process starts with direct interaction of TF IIIA with a 5S rRNA gene, followed by sequential binding of two other factors, TF IIIC and TF IIIB, and finally RNA polymerase III [1–6]. Unlike the last two peptides which are required for the synthesis of both tRNA and 5S rRNA, TF IIIA appears to be required exclusively for 5S rRNA synthesis. It has been shown that *Xenopus laevis* TF IIIA binds specifically to the internal control region (ICR) of the 5S rRNA gene. Footprinting experiments showed that the protein shielded nucleotides +45 to +96 of the gene from DNase I digestion [1, 2].

In addition to the DNA transcriptional activities, TF IIIA also shows a 5S rRNA binding capability. It forms a 7S ribonucleoprotein particle (RNP) which functions in the cytoplasm of immature *X. laevis* oocytes to store RNA for ribosome assembly [2, 7]. TF IIIA with molecular mass of about 40 kDa is the best characterized eukaryotic transcription factor known to date. An analysis of its primary structure deduced from cDNA sequence, together with zinc requirements for DNA binding [8, 9] suggested the presence of "zinc finger" domains in TF IIIA [10]. The same motif has been found in other proteins and was postulated to be a prototype of a variety of eukaryotic DNA binding peptides [11–13]. Because of the role of TF IIIA in 5S

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Abbreviations: BB, bromophenol blue; BSA, bovine serum albumin; DTT, dithiothreitol; ICR, internal control region; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethylsulphonyl fluoride; TF IIIA, transcription factor IIIA; 5S rDNA, 5S rRNA gene; 7S RNP, 7S ribonucleoprotein particle.

rRNA gene transcription, its function as a model zinc finger protein and its unusual properties as a sequence-specific DNA- and RNA-binding protein, transcription factor IIIA is of great interest.

In contrast to the TF IIIA from *X. laevis,* relatively small progress has been made in the studies on other eukaryotic transcription factors. This is probably due to difficulties with purification, owing to their low concentration in somatic cells [14]. However, TF IIIA from yeast, insect, HeLa cell extracts and from frogs has been purified [15–19]. Generally, these proteins are similar to that of *X. laevis* in respect of their function but not in their amino-acid sequences.

Until now, no TF IIIA-type protein has been characterized in plants. For better understanding of the mechanism of transcription of pol III genes in plants and interactions of plant 5S rRNA with protein, homologous TF IIIA is needed. Recently, on the basis of a variety of biochemical and biophysical data, some of us [20] presented evidence for a new tertiary structure model of plant 5S rRNA [21], with long range tertiary interactions between the loops C and D, and B and E, respectively [22]. The data suggested that 5S rRNA has an elongated shape when complexed with TF IIIA or with some of its fragments [23]. In order to study the mechanism of RNA-protein interactions, pure homologous molecules are required. The objective of the present work was to purify and characterize a plant protein, which binds mature plant 5S rRNA as well as 5S rRNA genes. Here, for the first time we describe the isolation and characterization of the transcription factor of a TF IIIA type from Gramineae.

MATERIALS AND METHODS

Purification of plant TF IIIA. The protein was isolated from the pollen of S 215 inbred line of maize (*Zea mays* L.). The tissue was collected at the Plant Breeding Station Smolice and stored at –70°C. The pollen (6 g) was homogenized and suspended in buffer A (20 mM Mes, pH 6.0, 15 mM NaN_3, 2 mM $MgCl_2$, 1 mM DTT, 1 mM benzamidine and 0.5 mM PMSF) on ice. The crude extract was centrifuged at 40000 r.p.m. for 60 min in a Beckman centrifuge rotor 70.1 Ti at 4°C. Three ml of the supernatant (A_{260} 500) concentrated on Amicon filtration apparatus was applied for a Sephadex G-50 column (1.5 cm × 100 cm) equilibrated with buffer A. Fractions of 1 ml were collected at a flow rate of 6 ml/h. Protein fractions 40–70 from the Sephadex G-50 column were pooled and loaded on a DEAE-Sepharose CL 6B (2.6 cm × 32 cm) column. The chromatography was carried out at 4°C using a linear gradient of 0–0.35 and 0.35–0.5 M NaCl in buffer A. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h.

SDS-polyacrylamide gel electrophoresis. The gel electrophoresis (SDS/PAGE) of proteins was performed as described by Laemmli [24]. The samples were dissolved with 2 vol. of sample buffer (25 mM Tris, pH 6.8, 0.5% SDS, 5% glycerol, 0.05% bromophenol blue and 0.5% 2-mercaptoethanol). Electrophoresis was run on 15% polyacrylamide separating gel with a 4% stacking gel at 120 V. Molecular mass standards were bought from Pharmacia. Staining was performed with Coomassie brillant blue R-250 [25].

5S rRNA binding assay. The protein samples (5 µl of fractions from Sepharose CL 6B or 0.42–3 nM of concentrated protein) were used for binding to 1×10^{-9} M (10000 c.p.m.) of 3'-^{32}P-labeled *Lupinus luteus* 5S rRNA [26]. The reaction was carried out in buffer B (20 mM Hepes, pH 7.8, 100 mM KCl, 1 mM $MgCl_2$, 25 µM $ZnCl_2$, 5 mM DTT, 100 µg/ml BSA) for 30 min at room temperature (20°C). The complex formation was analysed on 0.7% agarose gel. In the competition assay, protein samples were incubated with *L. luteus* 5S rRNA and increasing concentration of unlabeled *L. luteus* $tRNA^{His}$ (4.5×10^{-10}–10^{-4} M) in the same conditions.

5S rDNA binding reaction. The fractions from DEAE-Sepharose CL 6B (5 µl) were used for binding to labeled *L. luteus* 5S rDNA. The assay was carried out in buffer C (20 mM Tris/HCl, pH 7.5, 2 mM $MgCl_2$, 70 mM KCl, 50 mM $Zn(OOCCH_3)_2$, 0.5 mM DTT, 0.1 % Nonidet P-40) for 30 min at room temperature. A 3'-^{32}P-labeled 380 bp

*Eco*RI–*Hin*dIII DNA fragment (1×10^{-9} M, 10000 c.p.m.) containing *L. luteus* 5S rRNA gene [27] was used for each reaction. The complex formation analysis was done on 0.7% agarose gel.

DNA binding activity was measured using the following amounts of the concentrated maize protein: 0.65, 2.6, 6, 8 nM per a fixed amount (1×10^{-9} M, 10 000 c.p.m.) of 5′-^{32}P-labeled 120 bp *L. luteus* 5S rRNA gene, obtained by PCR. The binding reaction was carried out in buffer D (20 mM Tris/HCl, pH 7.4, 70 mM KCl, 2 mM $MgCl_2$, 20 μM $Zn(OOCCH_3)_2$, 0.2 mM DTT, 0.1% Nonidet P-40, 100 μg/ml BSA, 6% glycerol) for 30 min at room temperature.

Agarose gels. The analysis of the TF IIIA/5S rRNA and TF IIIA/5S rDNA complexes formation was carried out on 0.7% agarose gels. The samples were loaded on the gel in 4 mM Tris/HCl, pH 7.4 containing 4% glycerol and 0.02% bromophenol blue (BB) and run at 30 mA for 45 min (BB to the bottom of the gel) in 1 × TB buffer (45 mM Tris/boric acid, pH 8.3). The gel was dried under vacuum at 60°C and exposed to X-ray film.

Labeling of nucleic acids. 5S rRNA of *L. luteus* was purified as described previously [26] then it was labeled at 3′ end with [^{32}P]pCp (3000 Ci/mmol) (Amersham) and T4 ligase (Pharmacia) and purified on 10% polyacrylamide gel with 7 M urea [28, 29].

5S rRNA gene (5S rDNA) of *L. luteus* was obtained from plasmid pU5SLL (pUC 18 derivative containing a single copy of a *L. luteus* 5S rRNA gene [27]). A 380 bp fragment containing 5S rDNA was obtained after digestion with *Eco*RI and *Hin*dIII.

The *Eco*RI–*Hin*dIII fragment was labeled with [α-^{32}P]dATP (3000 Ci/mmol) (Amersham) and Klenow Fragment of DNA polymerase I (Pharmacia) [25], but the 120 bp PCR product corresponding to *L. luteus* 5S rDNA was labeled with [γ-^{32}P] ATP (3000 Ci/mmol) (Amersham) and T4 kinase (Pharmacia) [25].

Assay for transcriptional activity of plant TF IIIA. Standard assay contained in 20 μl: 6 μl of HeLa cell extract, 600 μM NTP (ATP, CTP, UTP), 10 mM creatin phosphate, 5 mM $MgCl_2$, 25 mM KCl, and 13.5 μM [α-^{32}P]GTP (440 Ci/mmol) (Amersham) and 0.04 μM 5S rDNA template (pU5SLL), 0.03 μM tobacco $tRNA^{Tyr}$ gene, 0.7 nM maize TF IIIA, 0.5 U RNasin and 50 μM $ZnCl_2$, respectively. Incubation was carried out at 30°C for 60 min and terminated by addition of 40 μl of stop buffer (100 mM sodium acetate, pH 5.5, 30 mM EDTA, 1 mg/ml poly A). The reaction mixture was extracted twice with 2 vol. of saturated phenol and phenol/chloroform. RNA was precipitated with 2.5 vol. of 95% cold ethanol, and the pellet was dried and resuspended in 10 μl of deionized formamide solution containing 0.1% each bromophenol blue and xylene cyanol. The sample was heated to 95°C for 3 min prior to loading onto 10% polyacrylamide, 7 M urea, 1 × TBE (89 mM Tris, 89 mM borate, 2.5 mM EDTA). After electrophoresis for 1.5 h at 40 mA, the gel was fixed in 10% acetic acid and 20% ethanol for 30 min and dried prior to autoradiography.

The association constant (K_a) of plant TF IIIA/nucleic acids and transcription level were estimated by autoradiogram densitometric analysis (LKB UltroScan XL).

RESULTS

The selected protein fractions showing TF IIIA activity obtained by a Sephadex G-50 column chromatography, as described under Materials and Methods, were pooled and further purified on a DEAE-Sepharose CL 6B column (Fig. 1A). The eluted fractions were analyzed for binding activity with 5S rRNA and 5S rRNA gene (5S rDNA) from *L. luteus* (Fig. 1B, C). The fractions 28–32 eluted at approximately 0.15 M NaCl showed nucleic acids binding affinity. They were pooled, dialyzed and concentrated. The Coomassie brillant blue stained SDS gel showed the pure band of maize TF IIIA with an apparent molecular mass of 35 kDa (Fig. 2, 3).

The isolated protein ($0.65–8 \times 10^{-9}$M) was analyzed for its ability to form a specific complex with plant 5S rRNA gene (Fig. 4).

Formation of plant 7S RNP was observed in the presence of increasing maize TF IIIA concentration ($0.42–3 \times 10^{-9}$ M) and 1×10^{-9} M of *L. luteus* 5S rRNA (Fig. 5). A 100% yield of the complex was obtained in

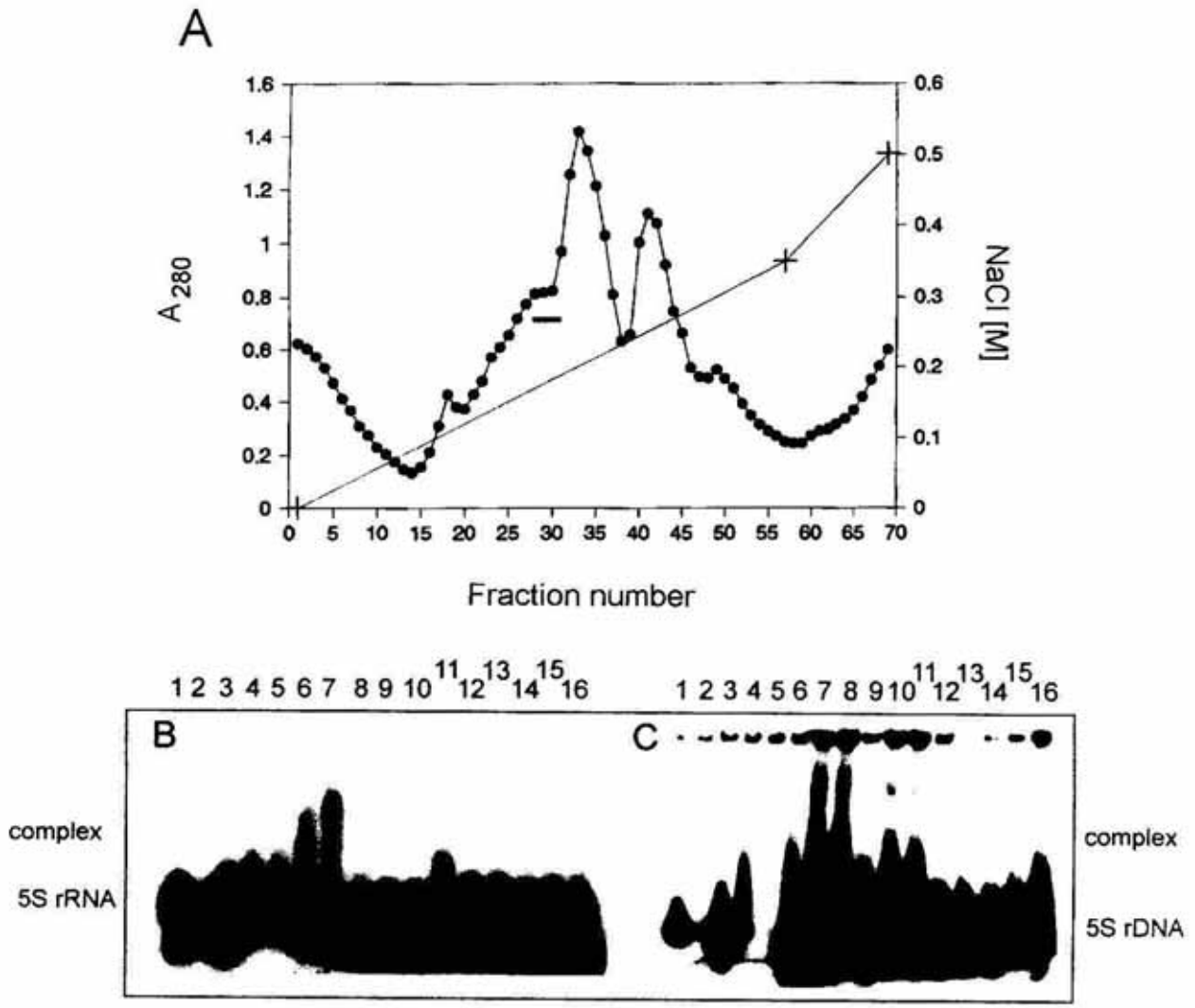

Figure 1. Purification of transcription factor IIIA from pollen grains of maize.

A. DEAE-Sepharose CL 6B column (2.6 cm × 32 cm) chromatography of protein extract after speed centrifugation of homogenized 6 g of pollen grains of maize and Sephadex G-50 (1.5 cm × 100 cm) filtration. Elution was followed by measuring the absorbance at 280 nm. Elution was done with 0–0.35 and 0.35–0.5 M NaCl gradient in the same buffer. Fractions of 0.5 ml were collected at a flow rate of 3 ml/h. The straight line represents NaCl gradient. **B** and **C**. Autoradiogram of 5S rRNA (**B**) and 5S rDNA (**C**) binding activity of the proteins from maize pollen. Analysis was carried out in 0.7% agarose gel. Lane 1, *Lupinus luteus* 5S rRNA (**B**) or 5S rDNA (**C**) in buffer B and C, respectively, lanes 2–16 5 µl of fractions No. 1, 4, 18, 20, 24, 28, 36, 40, 42, 46, 48, 50, 62, 66 containing 5S rRNA (**B**) and 5S rDNA (**C**), respectively.

the presence of 2.9×10^{-9} M maize TF IIIA (Fig. 5, lane 8). Densitometric analysis of the autoradiogram showed that the association constant (K_a) of plant 7S RNP is of the order of 1×10^9 M^{-1} (Fig. 5). Experiments with 5S rDNA indicated that plant TF IIIA binds *L. luteus* 5S rRNA with the same K_a. This means that maize TF IIIA forms with lupin DNA and RNA complexes similar to that obtained with *X. laevis* TF IIIA.

Specificity of the binding of purified protein with 5S rRNA was studied in the presence of increasing concentration (4.5×10^{-4}–10^{-9} M) of unlabeled and unspecific RNA ($tRNA^{His}$) (Fig. 6). The competition effect was observed at an almost 50 000-fold excess of *L. luteus* tRNA over maize 5S rRNA (Fig. 6 lane 7).

The ability of the maize pollen TF IIIA to initiate *L. luteus* 5S rRNA gene transcription *in vitro* was proved in HeLa cell extract

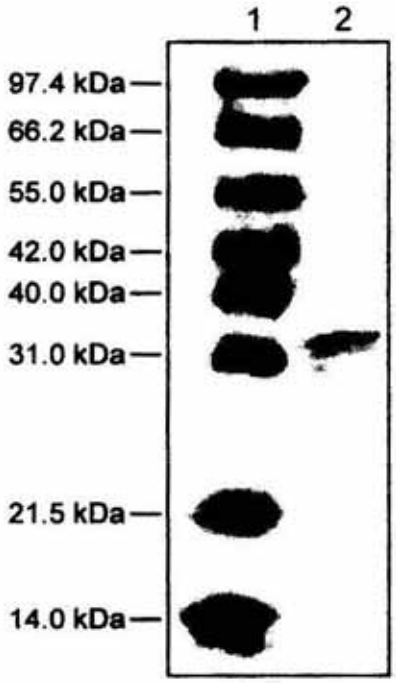

Figure 2. Electrophoresis on 15% polyacrylamide gel of the maize transcription factor TF IIIA.

Gel was stained with Coomassie brillant blue. Lane 1, molecular mass markers; lane 2, concentrated maize protein eluted from DEAE Sepharose CL 6B column.

(Fig. 7). Transcription of 5S rRNA was stimulated by the addition of 0.7 nM maize TF IIIA (Fig. 7A, B lanes 3, 6). In the presence of a ribonuclease inhibitor (RNasin) and 50 μM $ZnCl_2$ the level of 5S rRNA gene transcription was improved (Fig. 7A, B, lanes 4, 5, 7, 8). An analysis of autoradiograms of gels showed that plant TF IIIA significantly stimulated the activation of 5S rRNA gene showing optimum concentration at 0.04 μM (Fig. 7). The results clearly show that the plant 5S rRNA gene is transcribed in a crude HeLa cell extract in the presence of plant TF IIIA.

DISCUSSION

Eukaryotic 5S rRNAs are encoded by multigene families in which the number of 5S rRNA gene copies per genome varies from about 1000 to several thousand in *Arabidopsis thaliana* and mammals, respectively [30, 31]. Transcription of these genes is catalyzed by polymerase III in the presence of additional proteins. The protein responsible for

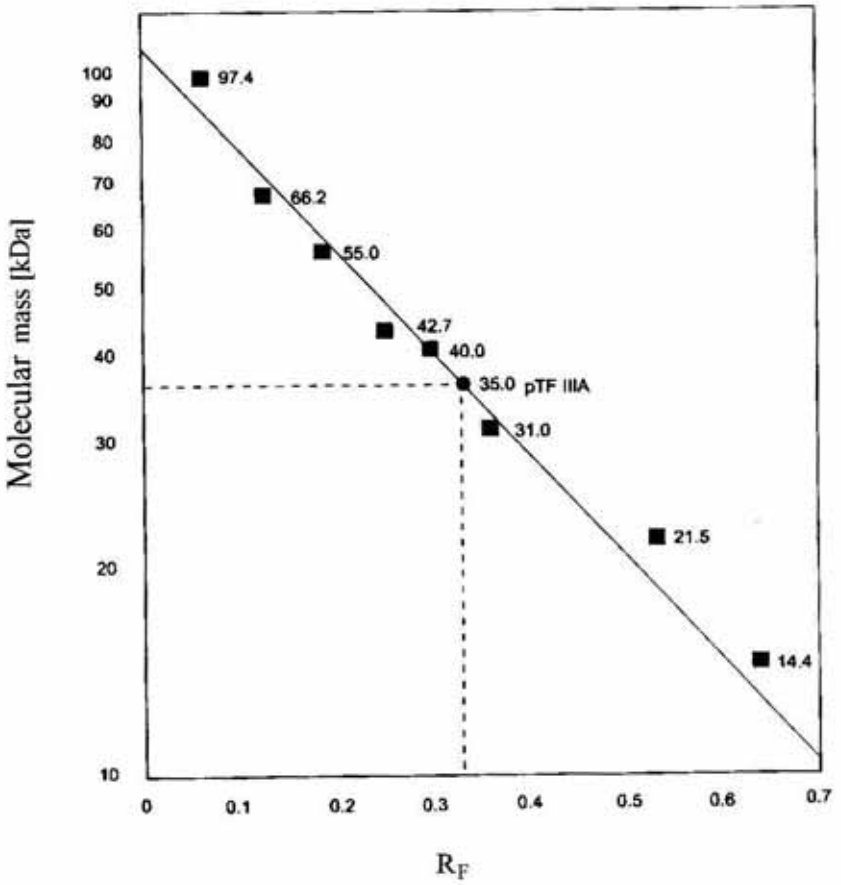

Figure 3. Determination of the molecular mass of maize pollen TF IIIA (pTF IIIA) by 15% SDS/PAGE analysis.

The relative mobility of indicated marker proteins was correlated with migration of plant protein.

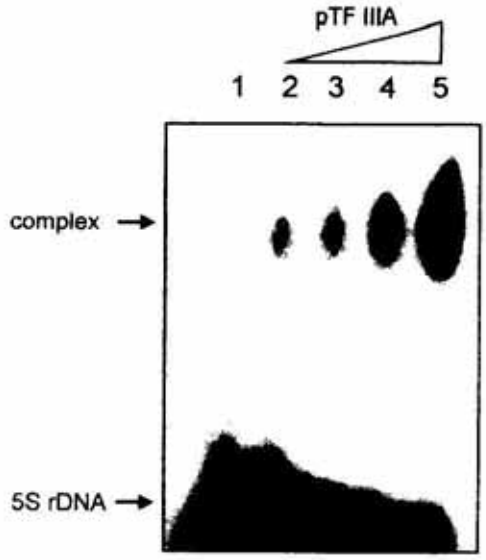

Figure 4. Autoradiogram of 5S rDNA binding activity of protein from pollen grains of maize.

Analysis was carried out in 0.7% agarose gel. Complex formation between 120 bp PCR product (5S rRNA gene coding sequence) and plant TF IIIA. Lane 1, *L. luteus* 5S rRNA gene in binding buffer D; lanes 2–5, complex formation with increasing plant protein concentration: 0.65, 2.6, 6, 8 $\times 10^{-9}$ M, respectively.

this process in *X. laevis* oocytes, called transcription factor IIIA (TF IIIA), has been isolated and characterized [32–34]. It shows the ability to bind both 5S rRNA gene and 5S rRNA molecules. Over the last several years various data on interactions between *X. laevis* TF IIIA and nucleic acids have been accumulated [35]. However, very little is known about the transcription of pol III genes in plants. The protein isolated from maize pollen resembles *X. laevis* TF IIIA in respect of its biochemical properties. The protein shows also 5S rRNA transcriptional activity. Our first attempts to purify TF IIIA from wheat germ and lupin were unsuccessful because of its low content in the tissues used for isolation. It is known that the accumulation of TF IIIA is developmentally regulated in *X. laevis* [2, 36]. Early in the oogenesis, TF IIIA is present at approximately 10^{12} molecules per cell thereby ensuring the continued activation of all 5S genes, accumulation of 5S rRNA and recruitment of TF IIIA into 7S RNPs. The cellular level of TF IIIA decreases late in oogenesis and then further from fertilization throughout embryogenesis [8]. In a HeLa cell there are 400 molecules of TF IIIA [18]. This is in agreement with the levels of TF IIIA present in somatic tissues of *X. laevis,* which are estimated at 3000 copies per cell in swimming tadpoles and

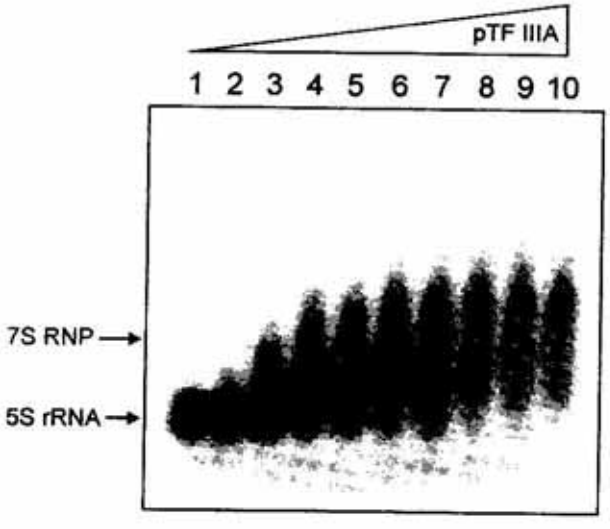

Figure 5. Autoradiogram of the *L. luteus* 5S rRNA binding activity of the protein from maize pollen.

Analysis was carried out on 0.7% agarose gel. Lane 1, *L. luteus* 5S rRNA in binding buffer B; lanes 2–10 complex formation at increasing concentration of maize protein 0.42, 0.84, 1.27, 1.68, 2.1, 2.5, 2.9, 2.95, 3 $\times 10^{-9}$ M, respectively.

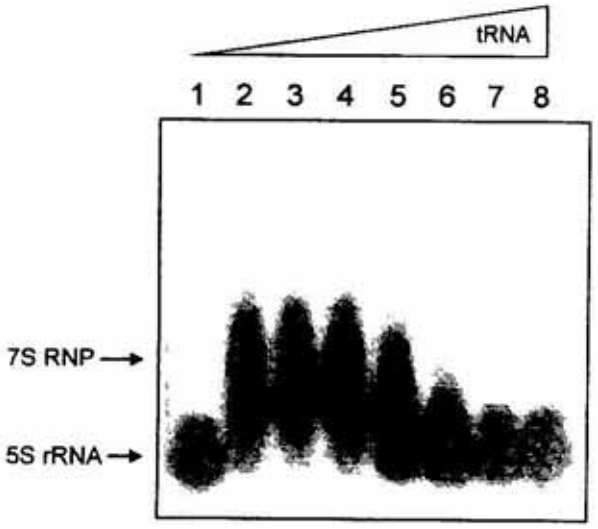

Figure 6. Autoradiogram of *L. luteus* 5S rRNA binding activity of maize TF IIIA in the presence of *L. luteus* $tRNA^{His}$ in increasing concentration.

Analysis was carried out on 0.7% agarose gel. Lane 1, *L. luteus* 5S rRNA in binding buffer B; lanes 2–8 complex formation between maize protein and 5S rRNA in the presence of tRNA, at the tRNA/5S rRNA ratio: 4.5:10, 4.5:1, 45:1, 4.5 $\times 10^{2}$:1, 4.5 $\times 10^{3}$:1, 4.5 $\times 10^{4}$:1, 4.5 $\times 10^{5}$:1, respectively.

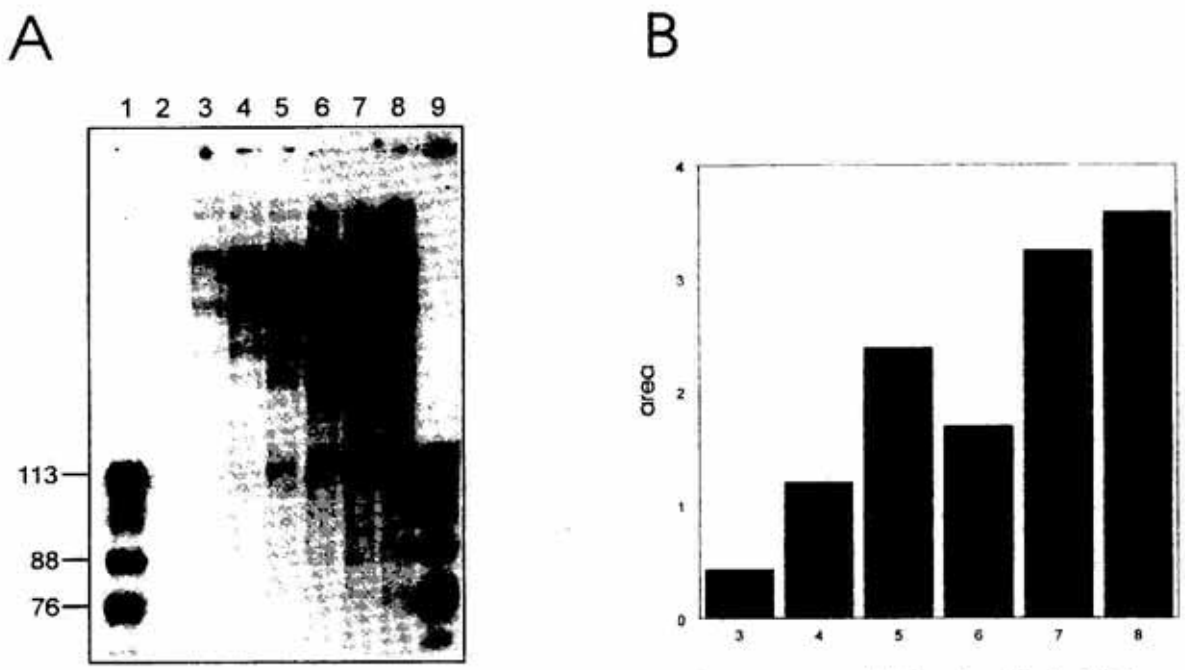

Figure 7. Stimulation by maize TF IIIA of *L. luteus* 5S rRNA gene transcription *in vitro* in HeLa cells extract.

A. Lane 1, control transcription of tobacco $tRNA^{Tyr}$ gene; lane 2, control transcription of homogenate 5S rRNA gene; lane 3, control transcription of *L. luteus* 5S rDNA; lane 4, control transcription of *L. luteus* 5S rDNA in the presence of 0.5 U RNasin; lane 5, control transcription of *L. luteus* 5S rDNA in the presence of 0.5 U RNasin and 50 μM $ZnCl_2$; lane 6, transcription of *L. luteus* 5S rDNA in the presence of 0.7 nM plant TF IIIA; lane 7, transcription of *L. luteus* 5S rDNA in the presence of 0.7 nM plant TF IIIA and 0.5 U RNasin; lane 8, transcription of *L. luteus* 5S rDNA in the presence of 0.7 nM plant TF IIIA and 0.5 U RNasin and 50 μM $ZnCl_2$. B. Diagram of area peaks of the autoradiogram (A) was made by LKB UltroScan XL.

8000 copies per cell in adult kidney cells [8, 36].

Thus, low somatic TF IIIA levels appear to be a common feature of vertebrate cells. It is generally accepted that regulatory proteins occur in cells in small amounts. Therefore, the main problem was to choose an appropriate tissue for isolation of plant TF IIIA. We checked many plant tissues but the best turned out to be pistils of tulip (unpublished) and maize pollen. Despite many efforts, so far neither a pol III transcription system nor a TF IIIA type protein has been isolated from plants. In this paper we describe for the first time the isolation of a maize protein with both 5S rRNA and 5S rRNA gene binding activities.

The protein with the molecular mass of 35 kDa binds to lupin 5S rRNA and 5S rDNA with a similar association constant (K_a) of 1×10^9 M^{-1}. TF IIIA of *X. laevis* has a similar affinity to 5S rRNA and 5S rRNA gene which are 1.4×10^9 M^{-1} and 1.9×10^9 M^{-1}, respectively [37, 38]. Maize TF IIIA binds *L. luteus* 5S rRNA with a high specificity. A competition effect was observed at an about 50000-fold molar excess of tRNA over 5S rRNA, e.g. TF IIIA from HeLa cells binds tRNA exactly at a 300-fold molar excess [39] on the other hand *X. laevis* TF IIIA has a 100 times greater affinity to 5S rRNA than to tRNA [38].

A comparison of the molecular mass of the earlier isolated TF IIIAs revealed that the factors vary from species to species, e.g. the 35 kDa plant protein is of the same size as its Hela cell (35 kDa) and silkworm (35 kDa) counterparts, but is smaller than the factor isolated from yeast (50 kDa) and frogs (40 kDa) [16, 17, 19, 40, 41].

Up to now, 5S rRNA transcription systems *in vitro* have been developed from *Acanthamoeba castelliani, X. laevis, Saccharomyces cerevisae, Bombyx mori, Caenorhabditis elegans, Aspergillus nidulans, Drosophila, Neurospora* and HeLa cells [42–50]. However, a pol III transcription system which facilitates accurate transcription of the RNA

polymerase II and III dependent U6 snRNA gene, has not been reconstructed from any plant except in *Nicotiana tabacum* [51]. In this work we used for transcription of 5S rRNA the HeLa cell extract because up to now there is no active plant pol III transcription assay [52]. Analysis of autoradiograms of transcription products shows that maize TF IIIA stimulates the transcription of 5S rRNA gene at least two fold when compared with the same reaction without the plant protein. In the presence of RNasin and $ZnCl_2$ an up to 3.8-fold increase of the transcription level was observed (Fig. 7 A, B). It is possible that the lower level of transcription is due to the presence of ribonucleases in HeLa cell extract.

Low specificity of interactions of plant TF IIIA with transcription factors and polymerase III from HeLa cells as well as inhibition of transcription by other not known plant factors [52] could be the reason for the low level of this reaction. It seems that these results could reflect the structural differences in the interaction between the transcription factors from plants and mammals as well as between the transcribed genes. The genes for tRNA apparently require only a single transcription factor IIIC for the recognition of the promoter and formation of a metastable or stable transcription complex [53, 54]. It seems possible that plant and human factors could not be exchanged individually like the transcription factors of polymerase II from *Saccharomyces cerevisae* and *Schizosaccharomyces pombe* [55]. Two components, analogues of human transcription factors IIF (TF IIF) and IIH (TF IIH) could be exchanged individually between the systems without affecting the start site. However, three counterparts of human TF IIB, TF IIE and RNA polymerase II could not be exchanged individually, but could be swapped in pairs TF IIE-TF IIH and TF IIB-RNA polymerase II, this demonstrates that there are functional interactions between these components [55].

Finally, both the DNA and RNA binding properties of maize TF IIIA presented here points to functional similarities of plant and amphibian factors which may reflect similarities between their protein structures.

Future experiments involving cloning of the gene for the plant transcription factor are in progress.

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