Domain Organization and Functional Properties of Yeast Transcription Factor IIIA Species with Different Zinc Stoichiometries*

(Received for publication, April 21, 1998, and in revised form, October 16, 1998)

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Transcription factor IIIA (TFIIIA) binds to the 5 S rRNA gene through its zinc finger domain and directs the assembly of a multiprotein complex that promotes transcription initiation by RNA polymerase III. Limited proteolysis of TFIIIA forms with different zinc stoichiometries, in combination with DNA binding and in vitro transcription analyses, have been used herein to investigate the domain organization and zinc requirements of Saccharomyces cerevisiae TFIIIA. Species containing either nine, six, or three zinc equivalents were produced by reductive resaturation and controlled metal depletion of recombinant TFIIIA. Partial digestion of the metal-saturated, 9 Zn²⁺-liganded factor yields a stable intermediate comprising the eight N-terminal zinc fingers, and a less stable fragment corresponding to a C-terminal portion including the ninth finger. Proteolyzed TFIIIA has the same 5 S DNA binding ability of the intact protein yet no longer supports in vitro 5 S rRNA synthesis. Both the structural compactness and the 5 S DNA binding ability of the TFIIIA form only containing 3 zinc ions are severely compromised. In contrast, the 6 Zn²⁺-liganded species was found to be indistinguishable from metal-saturated TFIIIA. By demonstrating the existence of three classes of zinc-binding sites contributing differently to yeast TFIIIA structure and function, the present study provides new evidence for the remarkable flexibility built into this complex transcription factor.

* This work was supported by grants from the National Research Council of Italy (CNR), Target Project on “Biotechnology,” and from the Ministry of University and of Scientific and Technological Research (MURST, Rome, Italy). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: TF, transcription factor; ICR, internal control region; TBP, TATA box-binding protein; r, recombinant; PAR, 4-(2-pyridylazo)resorcinol; PMPS, p-hydroxymercuropieryl sulfonate; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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does not constitute 5 S rRNA synthesis in a yeast in vitro transcription system (32), makes the yeast protein a particularly interesting candidate for detailed structural and functional studies.

Among the various strategies that have been employed to study the functional role of individual TFIIIA fingers and to understand the reasons for such an extended DNA-binding surface, the analysis of deletion and so-called "broken finger" mutants has been the most widely used. Whereas the first approach makes use of artificially truncated polypeptides, with the inherent risk of anomalous structural rearrangements, the second approach allows, in principle, the disruption of individual zinc fingers without introducing any major alteration in the overall structure of the protein. These two complementary approaches have contributed to elucidate the topography of individual finger-DNA interactions on the 5 S RNA gene as well as the contribution of different fingers to DNA or RNA binding (reviewed in Refs. 6 and 24). More recently, through the combined use of these two approaches, it has been inferred that the interaction between TFIIIA and 5 S DNA is a complex one, in which all fingers can be equally involved in 5 S DNA binding (without the thermodynamic dominance of any specific subset of fingers) and that functional interactions between fingers significantly contribute to the overall binding energy (33). In keeping with the complex mode of binding emerging from these studies, the solution structure of the first three zinc fingers of Xenopus TFIIIA complexed with 5 S DNA also evidenced an important role of finger-finger interactions in determining specific DNA binding (34, 35), and the recently solved crystal structure of the first six zinc fingers of Xenopus TFIIIA bound to the cognate DNA revealed that different fingers can contact DNA in a very different manner (36, 37). Given the complexity of the TFIIIA DNA-binding domain and the functional interdependence of individual fingers, it would be most informative to address the overall organization of TFIIIA by changing the zinc coordination state of the protein without introducing any amino acid sequence alteration. Such a strategy could be based on the stepwise removal of zinc ions from metal- saturated TFIIIA. The existence of distinct classes of zinc-binding sites with different metal affinities has been documented previously for Xenopus TFIIIA (38, 39), yet the structural and functional consequences of partial zinc removal have not been examined, and nothing is known about the significance of this heterogeneity in zinc binding affinity.

By taking advantage of the availability of transcriptionally competent, purified recombinant yeast TFIIIA (15) and of a highly resolved in vitro transcription system (40), we set out to investigate, with a non-mutagenic approach, the role of zinc ions in determining the properties of the yeast transcription factor. We first determined the native domain organization of the protein by means of limited proteolysis. TFIIIA forms with different zinc stoichiometries were then generated and characterized by expression from a structural and functional point of view.

**EXPERIMENTAL PROCEDURES**

**Yeast TFIIIA Expression and Purification—**Yeast TFIIIA was expressed at high levels in Escherichia coli BL21(DE3) cells co-transformed with plasmid pET-IIIA and a multicopy plasmid carrying the gene for a minor tRNA^{psi}(AGA/AGG) species (15). Recombinant TFIIIA purification followed a previously described procedure (15) with a few modifications. One of these was the introduction of a nucleic acids removal step at the beginning of the purification procedure. Accordingly, the pellet obtained from the initial ammonium sulfate precipitation (50% (NH_4)_2SO_4 saturation) of the soluble lysate obtained from 2 liters of bacterial culture was dissolved in 30 ml of TEGZ-β buffer (50 mM Tris-HCl, pH 7.5, 50 μM EDTA, 10% glycerol, 50 mM ZnSO_4, 2 mM β-mercaptoethanol), dialyzed for 4 h against the same buffer containing 0.6 M NaCl (TEGZ-β0), and centrifuged at 12,000 × g for 15 min. After adjustment to final NaCl and protein concentrations of 0.25 M and 0.75 mg/ml with TEGZ-β buffer, the clarified supernatant (360 ml) was adsorbed in batch to 50 ml of DEAE-Sepharose (Amersham Pharmacia Biotech) equilibrated in TEGZ-β0.25 buffer, and unbound proteins were then directly subjected to Bio-Rex 70 (Bio-Rad) chromatography. Other modifications were the elimination of β-mercaptoethanol from all buffers used after Bio-Rex chromatography and the introduction of a final purification step, carried out on a heparin Ultragel A4R (IBF Biotechnics) column (1 ml) equilibrated and washed with HNG buffer (30 mM Hepes-Na, pH 8.0, 10% glycerol) containing 0.1 M NaCl (HNG/0.1) and eluted with a 0.3–1 M NaCl gradient. Pooled fractions from heparin Ultragel chromatography contained yeast TFIIIA at greater than 90% homogeneity and were subsequently concentrated to a final volume of 7 ml, corresponding to a protein concentration of 1.25 mg/ml. During the initial phases of purification, protein concentrations were determined with the method of Bradford (41), whereas an ε_{280} value of 27,100 l mol^{-1} cm^{-1} (15) was used to determine TFIIIA concentration in highly purified samples.

**Limited Proteolysis of TFIIIA—**Samples of TFIIIA (3 μg each) were digested (37 °C) with trypsin (Sigma, T8642) in a final volume of 10 μl, at a 1:1000 protease/TFIIIA (w/w) ratio, in trypsinolysis buffer (30 mM Hepes-Na, pH 8.0, 100 mM NaCl, 8% glycerol). Identical amounts of TFIIIA were used for S. aureus V8 protease (Fluka, 45712) digestions (15–60 min, 37 °C), which were conducted in PBS buffer (80 mM NaHPO_4, 25 mM NaH_2PO_4, 100 mM NaCl, 8% glycerol) at a 1.5 protease/TFIIIA (w/w) ratio. Digestions were blocked with either the addition of 1 mM AEBSF (Sigma) or by a 2 min boiling in SDS-PAGE loading buffer. Proteolysis products were resolved by SDS-PAGE (42), visualized by Coomassie Blue staining, and quantitated by densitometric analysis carried out on digitized images with the Multi-Analyzer/PC software (Bio-Rad).

**N-terminal Sequencing of Protease-resistant Polypeptides—**Samples of TFIIIA (9 μg each) were digested with either trypsin or V8 protease under standard limited proteolysis conditions (30 min at 37 °C). The resulting polypeptides were resolved by SDS-PAGE (42), visualized by Coomassie Blue staining, and quantitated by densitometric analysis carried out on digitized images with the Multi-Analyzer/PC software (Bio-Rad).

**DNA Binding Assays—**For electrophoretic mobility shift assays, a 450-base pair DNA fragment, containing the yeast 5 S RNA gene, was prepared with plasmid pUC9-SS (a kind gift of B. H. H. T. Mahy, Ecole Polytechnique, Brussels) digested (37 °C) with HindIII digestion and end-labeled with [α-32P]dCTP by using polynucleotide kinase. After labeling and purification, DNA fragment concentration was determined with the method of Bradford (41), whereas an ε_{280} value of 27,100 l mol^{-1} cm^{-1} (15) was used to determine TFIIIA concentration in highly purified samples.

**Yeast Transcription Assays—**Template DNA was the yeast 5 S RNA gene contained in plasmid pUC9-SS. 250 ng of pUC9-SS were preincubated at 20 °C for 30 min in a 45-μl reaction mixture (30 mM Hepes-KOH, pH 7.9, 100 mM KCl, 5 mM MgCl_2, 8% glycerol, 8 units of RNasin (Ambion), 1 mM DTT) containing an internally balanced set of complementing fractions as follows: 20 ng of recombinant TBP (46), 60

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ng of recombinant TFIIIB70 (46), and 0.5 μg of B’ fraction (47), which together reconstitute TFIIIB activity, and 5 μg of a glycerol gradient fraction containing partially purified TFIIIC and RNA polymerase III (45). Following preincubation, RNA synthesis was started by adding 5 μl of a solution providing ATP, CTP, and GTP to a final concentration of 500 μM UTP to a final concentration of 25 μM, and 10 μCi of [α-32P]UTP (800 Ci/mmol, Amersham Pharmacia Biotech). Multiple rounds of transcription were allowed to proceed for 10 min at 20 °C; reaction products were then purified, resolved, and quantified as described (15). For single round transcription experiments, 5 S RNA was first preincubated with TFIIIC, TFIIIB, RNA polymerase III and limiting concentrations of TFIIIA for 30 min at 20 °C, in a volume of 50 μl. Stabilized elongation complexes paused at nucleotide 10 (48, 49) were then allowed to form by adding 5 μl of a solution supplying CTP and GTP to a final concentration of 500 μM, UTP to 25 μM, and 10 μCi of [α-32P]UTP. After 10 min at 20 °C, 2 μl of a solution providing 500 μM ATP and 300 μg/ml heparin (Sigma, H2149 type) were added to complete the synthesis of initiated transcripts under conditions of blocked reinitiation. The number of transcripts synthesized in a single round of transcription was taken to equal the number of active transcription complexes formed during preincubation, i.e. the number of active TFIIIA molecules. 5 S RNA transcripts were quantified from phosphorimages of dried gels obtained with a Personal Molecular Imager FX (Bio-Rad) using the Multi-Analyzer/PC software (Bio-Rad). The number of transcripts corresponding to each phosphorimaging signal was determined by comparison with the signal produced by a known amount of [α-32P]UTP. All solutions used for in vitro transcription assays were made with Chelex-treated, double-distilled water and ultrapure reagents. Preparation of Yeast TFIIIA Species with Different Zinc Stoichiometries—Zinc-saturated TFIIIA was prepared by incubating the purified protein (1.25 mg/ml) for 1 h at 20 °C in the presence of 5 mM DTT, followed by a 4-h dialysis against HNG/0.35 buffer containing 50 μM ZnSO4, and by the final removal of unbound zinc with a 20-h dialysis against the same buffer without added zinc. The 6 Zn2+-liganded TFIIIA species was obtained upon incubation of metal-saturated TFIIIA (1 h, 20 °C) in the presence of 0.5 mM 4-(2-pyridylazo)resorcinol (PAR, Sigma) and removal of excess reagent by a 20-h dialysis (two changes) against the same buffer without added zinc. The 6 Zn2+-liganded TFIIIA species was similarly prepared by incubation (30 min, 20 °C) with 2 mM EDTA, followed by the removal of excess EDTA with a 20-h dialysis (two changes) against HNG/0.5 buffer (an increased salt concentration). TFIIIA species containing 3 zinc ligands was similarly prepared by dialysis against 0.1 mM PAR, and removal of excess reagent by a 20-h dialysis (two changes) against HNG/0.35 buffer containing 0.1 mM PAR, adjusting the spectrophotometer zero absorbance immediately before protein addition; higher absorbance changes were used for absorbance changes at 500 or 250 nm (in the case of tinctorial changes in the absence of PAR) were measured after each PMPS addition. Zinc release in the presence of PAR only was similarly analyzed by measuring absorbance changes at 500 nm as a function of time. Zinc release from TFIIIA was determined indirectly by monitoring the formation of a (PAR)Zn2+ complex (ε350 = 66,000 cm−1 M−1), whereas mercaptoimidazole bond formation was directly followed at 250 nm. PMPS and PAR stock solutions (4 and 5 mM, respectively) were prepared with Chelex-treated buffers as described previously (44, 49). For the determination of the zinc content of buffers and reaction mixtures, PAR was added to a final concentration of 0.1 mM, and the decrease in absorbance at 500 nm was measured after the addition of 1 mM EDTA. All spectrophotometric measurements were conducted with a Cary 1B spectrophotometer. RESULTS Limited Proteolysis of Yeast TFIIIA—We initially probed the structural organization of yeast TFIIIA by limited proteolysis. Transcriptionally competent recombinant yeast TFIIIA, purified from an overproducing E. coli strain, and either trypsin or V8 protease were utilized for this analysis. As shown in Fig. 1A, a 33.5-kDa proteolytic fragment (p33.5) was already clearly detectable after a 10-min incubation in the presence of trypsin (lane 2); it became the main product of trypsinolysis following an additional 20-min digestion that led to the complete disappearance of full-length TFIIIA (lane 4) and remained stable even after a 1-h incubation in the presence of trypsin (data not shown). A smaller and less represented fragment of about 15 kDa (p15) was generated at the same time as p33.5 (lane 2) and decreased upon further digestion, concomitantly with the appearance of an 11.5-kDa fragment (p11.5, lane 4). Two other minor fragments of 17 kDa (p17) and 30.5 kDa (p30.5) first appeared after 20 min of trypsinolysis (lane 3) and slowly accumulated thereafter. Fig. 1B shows that this trypsin cleavage pattern was not modified by the addition of excess zinc to the proteolysis mixture (cf. lanes 2 and 3). In contrast, the addition of a 100-fold
molar excess of EDTA (500 μM) prior to trypsin digestion caused a complete loss of the 33.5-kDa fragment and of all the other smaller polypeptides (Fig. 1B, cf. lanes 2 and 4). The residual undigested protein observed in this latter case likely results from nonspecific self-aggregation of zinc-depleted TFIIIA. We interpreted the destabilizing effect of EDTA as the result of a conformational alteration of TFIIIA induced by zinc removal. Indeed, protease resistance was fully restored by a 10-min incubation of EDTA-treated TFIIIA with 500 μM Zn²⁺ prior to proteolysis (Fig. 1B, lane 5).

In yeast TFIIIA there are 62 potential trypsin cleavage sites (lysine and arginine) and 54 additional sites for the V8 protease (glutamate and aspartate), yet the latter enzyme also generated, as the main product, a stable, zinc-dependent intermediate of 34.5 kDa (data not shown), thus confirming the existence in TFIIIA of region(s) that in the native protein are especially accessible to proteases.

To define the boundaries of the protease-resistant regions of yeast TFIIIA, we next determined the N-terminal sequence of the polypeptide fragments generated by either trypsin or V8 protease and inferred the positions of the corresponding C-terminal cleavage sites by matching the size of each fragment with the occurrence of downstream cleavage sites for either enzyme. As summarized by the data reported in Fig. 2, the main products of partial digestion generated by the two proteases essentially correspond to the same polypeptide fragment. The 33.5-kDa, trypsinolysis fragment is generated by cleavage at Arg³⁹ and ends in a region, centered around Arg²³⁵, containing five contiguous lysine and arginine residues; a corresponding 34.5-kDa polypeptide fragment, formed by cleavage at either Glu²⁸ or Glu³¹ (two N-terminal sites that are cleaved with equal efficiencies) and ending in a region centered around Glu²³⁷, was generated by V8 protease. The N-terminal sequence of all the smaller-sized fragments generated by trypsin was also determined. The p15 fragment starts at Lys³⁲⁸ and thus represents the C-terminal portion of TFIIIA that is missing from the 33.5-kDa polypeptide. Due to a gel artifact, the molecular mass measured for this fragment is higher than the size of 12.8 kDa that would be expected for a polypeptide comprised between Lys³²⁸ and the C terminus. The p11.5 polypeptide also starts at Lys³²⁸, indicating that this late appearing fragment results from further C-terminal cleavage of p15. Similarly, p30.5 and p17 represent secondary cleavage products of the 33.5-kDa polypeptide. The p30.5 fragment, which has the same N terminus as p33.5, is generated from it through further C-terminal digestion, whereas the p17 fragment, starting at Arg¹⁸⁰ within the fifth finger, is likely the product of a second cleavage event at the N terminus.

In its native state, yeast TFIIIA thus appears to be constituted by a highly structured, protease-resistant domain comprising the first eight zinc fingers (p33.5 in Fig. 1) and a small, less stable C-terminal domain comprising the last finger and about half of the 81-amino acid linker interposed between fingers 8 and 9 (p15 in Fig. 1). These two domains are connected by a flexible polypeptide region, centered on a cluster of basic amino acid residues, which is highly susceptible to proteolytic cleavage.

Functional Characterization of Partially Proteolyzed Yeast TFIIIA—Having defined the limited proteolysis pattern of yeast TFIIIA, we next analyzed the functional competence of
Zinc Requirements of Yeast TFIIIA

The partially proteolyzed protein by gel retardation and in vitro transcription assays. For these experiments, TFIIIA was first subjected to limited proteolysis; trypsin was then blocked with the specific inhibitor AEBSF, and upon verification of the complete conversion into the 33.5-kDa form by SDS-PAGE analysis, samples of the same reaction mixture were directly used for transcription reaction mixtures. Transcripts synthesized in reaction mixtures lacking exogenous TFIIIA, or supplemented with freshly thawed TFIIIA (15 ng), are shown for comparison in lane 1. The migration positions of the free DNA fragment (f), the TFIIIA-DNA complex (B), and the smaller sized p33.5-DNA complex (b) are indicated on the left. The more slowly migrating complex in lane 6, presumably resulting from DNA binding by a dimeric aggregate of proteolyzed TFIIIA, is marked by an asterisk. All the lanes shown come from the same exposure of a single gel. B, transcription analysis. The transcription capacity of proteolyzed TFIIIA was tested in an in vitro transcription system reconstituted from rTBP, rTFIIB70, B' fraction, and a fraction supplying both TFIIIC and RNA polymerase III activities, with the yeast 5 S rRNA gene as a template. Increasing amounts of TFIIIA (from 7.5 to 120 ng), incubated for 30 min at 37 °C in the absence (lanes 2–4) or in the presence (lanes 5–9) of trypsin, were added to transcription reaction mixtures. Transcripts synthesized in reaction mixtures lacking exogenous TFIIIA, or supplemented with freshly thawed TFIIIA (15 ng), are shown for comparison in lanes 1 and 12, respectively. Trypsin digestion was stopped by the addition of 1 mM AEBSF prior to in vitro transcription. Transcripts in lanes 10 and 11 (T/in) come from control reactions in which AEBSF-blocked trypsin was added to a reconstituted in vitro transcription system containing 15 ng of untreated TFIIIA. The position of transcripts (5 S RNA) after resolution on a polyacrylamide gel is shown.

Fig. 3. 5 S rRNA gene binding and transcriptional activation properties of partially proteolyzed TFIIIA. A, 5 S DNA binding. A 450-bp radioactively labeled DNA fragment containing the yeast 5 S rRNA gene (1 ng) was incubated, prior to non-denaturing gel electrophoresis, with the following protein samples: 10 ng of freshly thawed TFIIIA (A, lane 2); 10 ng of TFIIIA preincubated for 30 min at 37 °C in proteolysis buffer lacking trypsin (A', lane 3); increasing amounts of TFIIIA subjected to a 30-min trypsin digestion at 37 °C (10, 20, and 40 ng in lanes 4–6, respectively). Trypsin was blocked with 1 mM AEBSF before incubation with the DNA fragment; no protein was added to the sample in lane 1. The migration positions of the free DNA fragment (f), the TFIIIA-DNA complex (B), and the smaller sized p33.5-DNA complex (b) are indicated on the left. The more slowly migrating complex in lane 6, presumably resulting from DNA binding by a dimeric aggregate of proteolyzed TFIIIA, is marked by an asterisk. All the lanes shown come from the same exposure of a single gel. B, transcription analysis. The transcription capacity of proteolyzed TFIIIA was tested in an in vitro transcription system reconstituted from rTBP, rTFIIB70, B' fraction, and a fraction supplying both TFIIIC and RNA polymerase III activities, with the yeast 5 S rRNA gene as a template. Increasing amounts of TFIIIA (from 7.5 to 120 ng), incubated for 30 min at 37 °C in the absence (lanes 2–4) or in the presence (lanes 5–9) of trypsin, were added to transcription reaction mixtures. Transcripts synthesized in reaction mixtures lacking exogenous TFIIIA, or supplemented with freshly thawed TFIIIA (15 ng), are shown for comparison in lanes 1 and 12, respectively. Trypsin digestion was stopped by the addition of 1 mM AEBSF prior to in vitro transcription. Transcripts in lanes 10 and 11 (T/in) come from control reactions in which AEBSF-blocked trypsin was added to a reconstituted in vitro transcription system containing 15 ng of untreated TFIIIA. The position of transcripts (5 S RNA) after resolution on a polyacrylamide gel is shown.

In a parallel set of experiments (Fig. 3B), we tested the ability of partially proteolyzed TFIIIA to support 5 S RNA synthesis in a yeast in vitro transcription system consisting of recombinant TBP, recombinant TFIIIB70 plus a partially purified B' fraction (which together reconstitute TFIIIB activity (47)), and a glycerol gradient fraction contributing both TFIIIC and RNA polymerase III activities (45). In this system, only background levels of transcription, due to the presence of trace amounts of contaminating TFIIIA in the TFIIIC/RNA polymerase III fraction, could be detected in the absence of exogenously added TFIIIA (Fig. 3B, lane 1). The addition of increasing amounts of control TFIIIA (preincubated for 30 min at 37 °C in proteolysis buffer lacking trypsin) resulted in a large increase of transcription, and a plateau was reached upon supplementation of 30 ng of TFIIIA (lanes 2–4). In contrast, the addition of up to 120 ng of partially proteolyzed TFIIIA failed to raise transcription above the background level (lanes 5–9). The observed transcriptional impairment of partially proteolyzed TFIIIA was not due to the accidental degradation of other transcription components by unblocked trypsin, because the addition of pre-blocked trypsin to a fully reconstituted transcription system did not interfere with 5 S RNA synthesis (compare lanes 10 and 11 with lane 3).

We conclude from these data that the protease-resistant, eight zinc finger domain of yeast TFIIIA is fully competent for specific 5 S DNA binding yet unable to support 5 S RNA transcription. In keeping with the results of previous deletion mutant analyses (30, 31), the transcription activation function of yeast TFIIIA thus appears to be associated with a 100-amino acid long C-terminal domain (corresponding to the p15 polypeptide in Fig. 1) that includes the last finger and part of the linker region separating fingers 8 and 9.

Different Classes of Zinc-binding Sites in Yeast TFIIIA—To gain insight into the contribution of zinc ions to the conformational stability of yeast TFIIIA, we determined the metal content of unmodified rTFIIIA and of the EDTA-treated TFIIIA species previously found to be extremely sensitive to trypsin and V8 proteolysis. Zinc release from either form of TFIIIA was induced by chemical modification of cysteine residues with p-(hydroxymercuro)benzenesulfonate (PMPS) and was monitored in the presence of the metallochromic indicator 4-(2-pyridylazo)resorcinol (PAR) (44, 49). The release of protein-bound zinc ions was determined by measuring absorbance changes at 500 nm (the absorption maximum of the PAR-Zn²⁺ complex) resulting from the titration of TFIIIA with PMPS (Fig. 4A), whereas mercaptide bond formation between PMPS and TFIIIA cysteine residues was monitored at 250 nm in parallel titrations conducted with PMPS only (Fig. 4B). As shown in Fig. 4A, the titration of both forms of TFIIIA with PMPS resulted in a linear increase of absorbance, up to plateau values corresponding to the release of 7 and 3 zinc eq., respectively, from the untreated and the EDTA-treated species. Taken together with limited proteolysis data (Fig. 1), these results indicate that the presence of 7 zinc ions per protein molecule is sufficient to confer to yeast TFIIIA its distinctive protease resistance properties, whereas the removal of 4 additional zinc ions leads to a reversible destabilization of protein.
structure. Also apparent in Fig. 4 (cf. A and B) is the good correlation between $A_{500}$ and $A_{250}$ curves in the case of the untreated form, confirming that zinc release from TFIIIA was indeed due to cysteine modification. More specifically, the fact that break points in both curves occur at nearly identical PMPS equivalent values, corresponding to the release of 7 zinc ions and the titration of 14 reactive sulfhydryl groups, implies that, like in *Xenopus* TFIIIA (50), the release of 1 zinc ion from a yeast TFIIIA finger also requires the chemical modification of 2 cysteine residues. In fact, no such correlation between $A_{500}$ and $A_{250}$ curves was observed in the case of the EDTA-treated species (Fig. 4, cf. A and B), an observation that is best explained by the presence in this TFIIIA form of PMPS-reactive yet metal-uncoordinated cysteine residues.

At variance with the nine predicted zinc finger motifs that are recognizable in the polypeptide sequence of yeast TFIIIA, the above titration experiments only revealed 7 zinc ions/protein molecule, a stoichiometry that did not change following direct zinc supplementation (data not shown). Similarly, a total of 14 cysteine residues, instead of the expected 20, was found to be reactive with PMPS. We thus reasoned that both discrepancies may originate from the oxidation of a few cysteine residues during TFIIIA expression or purification (e.g. through mixed disulfide formation with exogenously supplied β-mercaptoethanol). Accordingly, a resaturation procedure was worked out, in which disulfide reduction by DTT is followed by the elimination of the reducing agent in the presence of excess zinc and the final removal of unbound zinc through anaerobic dialysis against Chelex-treated buffer. Data reported in Fig. 5 show that 9 zinc eq could indeed be recovered upon reductive resaturation with the above procedure and that this variation in the number of protein-bound zinc ions was accompanied by a corresponding increase of the number of PMPS-reactive cysteines (from 14 to 18 Cys residues). Interestingly, despite this variation in the number of protein-bound zinc ions, the trypsin sensitivity of metal-saturated TFIIIA remained the same as that previously determined for the 7 Zn$^{2+}$-liganded TFIIIA species (shown in Fig. 7; cf. lanes 2 and 3).

Another notable feature of the titration curve reported in Fig. 5A is the disproportionate release of 2 zinc eq (instead of the expected 0.5) upon the addition of the first PMPS equivalent. This may indicate that some zinc ions can be released from metal-saturated TFIIIA without any prior cysteine modification. Indeed, as shown in Fig. 6, approximately 2 Zn$^{2+}$ eq were very rapidly released from metal-saturated TFIIIA in the presence of PAR only, and a third one was more slowly released upon further incubation (30 min) in the presence of PAR. Under identical experimental conditions, only 1 zinc eq was slowly released from the EDTA-treated TFIIIA species (Fig. 5B, cf. A and B), an observation that is best explained by the presence of metal-uncoordinated cysteine residues in this TFIIIA form.

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**Fig. 4**. Determination of the zinc and sulfhydryl group content of untreated and EDTA-treated TFIIIA. **A**, zinc determination. Increasing amounts of PMPS were added to untreated TFIIIA (1 µM; open circles) or EDTA-treated TFIIIA (3 µM; open triangles) in Chelex-treated buffer containing 0.1 mM PAR. The spectrophotometer was adjusted to zero absorbance (500 nm) immediately before TFIIIA addition. Absorbance changes at 500 nm were monitored after each PMPS addition, and $DA_{500}$ values (normalized with respect to TFIIIA concentration) were plotted against the number of PMPS equivalents added. The number of released zinc equivalents was determined using an $e_{500}$ value of 66,000 M$^{-1}$ cm$^{-1}$ for the (PAR)$_2$Zn$^{2+}$ complex (see “Experimental Procedures” for details). **B**, sulfhydryl group determination. A PMPS titration of untreated and EDTA-treated TFIIIA (both at a concentration of 1 µM) was conducted as in A but in the absence of PAR. Absorbance changes at 250 nm, recorded after each PMPS addition, were plotted against the number of PMPS equivalents added.

**Fig. 5**. Zinc and sulfhydryl group content of metal-resaturated TFIIIA. Resaturated TFIIIA (1 µM) was titrated with PMPS in the presence (A) or in the absence (B) of PAR. Titration conditions were the same as in Fig. 4: $DA_{500}$ and $DA_{250}$ values were similarly plotted against the number of PMPS equivalents added.
released from non-saturated, 7 Zn\(^{2+}\)-liganded TFIIIA, implying that the 2 zinc ions missing from this TFIIIA species likely correspond to those that are rapidly released from metal-saturated TFIIIA. In keeping with this view, no zinc ion was “spontaneously” released from the EDTA-treated form of TFIIIA (Fig. 6).

Three zinc ions in metal-saturated yeast TFIIIA are thus associated to weak metal-binding sites. They can be removed without any prior cysteine modification, thereby generating a TFIIIA species carrying 6 zinc eq, which still exhibits the characteristic trypsin sensitivity of metal-saturated and 7 Zn\(^{2+}\)-liganded TFIIIA (Fig. 7, lanes 2–4). The apparent zinc dissociation constant of these three weak binding sites, estimated on the basis of the $K_d$ of the (PAR)\(_2\)Zn\(^{2+}\) complex (44), is higher than 10\(^{-7}\) M, whereas the zinc ions bound to the three sites of highest affinity, which are not released even in the presence of 2 mM EDTA, appear to be kinetically or thermodynamically trapped.

**Functional Properties of TFIIIA Species with Different Zinc Stoichiometries**—Having established conditions for the controlled metal depletion and resaturation of yeast TFIIIA, the three species thus generated, containing, respectively, 3, 6, and 9 zinc eq, were examined for their DNA binding and transcription activation capacities. The gel retardation assays reported in Fig. 8A show that the PAR-treated form, carrying 6 zinc eq, has a 5 S DNA binding ability indistinguishable from that of metal-saturated TFIIIA (cf. lanes 2–5 with lanes 6–9). Apparent dissociation constants for the TFIIIA-5 S DNA complex were next determined by incubating constant amounts of TFIIIA with varying concentrations of the 5 S DNA fragment (see “Experimental Procedures”). As shown in Table I, apparent $K_d$ values for metal-saturated and 6 Zn\(^{2+}\)-liganded TFIIIA differ by no more than 12% and are similar to the dissociation constant previously reported for the yeast TFIIIA-5 S DNA interaction (14). In contrast, 3 Zn\(^{2+}\)-liganded TFIIIA is severely impaired in its ability to bind the 5 S rRNA gene (cf. lanes 2–5 with lanes 10–13). No protein-DNA complex could be detected with this TFIIIA species under conditions in which metal-saturated TFIIIA shifted about half of the 5 S DNA fragment (cf. lanes 4 and 12 in Fig. 8A). A similar pattern was observed when testing the ability of these three TFIIIA forms to support *in vitro* transcription of the 5 S rRNA gene (Fig. 8B). The 6 Zn\(^{2+}\)-liganded species exhibited the same transcription activity of metal-saturated TFIIIA (cf. lanes 2–6 with lanes 8–12). In the case of the 3-zinc form, instead, low levels of 5 S rRNA synthesis could only be detected at the highest factor concentration (lanes 13–18), as if the large structural disorganization caused by the removal of 6 Zn\(^{2+}\) ions did not completely inactivate the protein. In doing these experiments, care was taken to avoid zinc rebinding to metal-saturated TFIIIA. To this end, all the buffers used for DNA binding and *in vitro* transcription assays were prepared with ultrapure reagents and Chelex-treated, double-distilled water, and the zinc content of 5× concentrated mock reaction mixtures (not containing TFIIIA) was evaluated with the same PAR procedure utilized for TFIIIA analysis. The amount of zinc thus determined corresponds to a concentration <6 mM in standard (1×) reaction mixtures. Based on a zinc dissociation constant higher than 10\(^{-7}\) M for the three weak binding sites and assuming that all this zinc is available to TFIIIA, it can be estimated that such zinc concentration would allow for the resaturation of less than 10% of the 6 Zn\(^{2+}\)-liganded TFIIIA molecules used for *in vitro* assays. We cannot exclude, on the other hand, that some contaminating zinc ions may be bound by the intermediate affinity sites present in 3 Zn\(^{2+}\)-liganded TFIIIA. The low residual activity exhibited by this particular TFIIIA species may thus be due to either incomplete inactivation or limited zinc rebinding.

Another potential artifact we considered is that loosely bound zinc ions, released from 9 Zn\(^{2+}\)-liganded TFIIIA in the presence of PAR, could derive from partially denatured, inactive molecules present in our TFIIIA preparations. According to this hypothesis, zinc release upon PAR treatment would all take place at the expense of these inactive molecules, leaving unchanged the zinc content as well as the trypsin resistance, DNA binding, and *in vitro* transcription properties of active TFIIIA molecules. To test this possibility, we determined the fraction of active molecules in different TFIIIA preparations by quantitative single round transcription assays (48) (see “Experimental Procedures” for details). We found that the fraction of transcriptionally active molecules ranged from 56 to 95% in four different preparations yielding TFIIIA species with 6.1 ± 0.3 zinc ions/molecule upon PAR treatment. This lack of correlation between the fractional activity and the zinc release capacity of different TFIIIA preparations argues that 3 loosely bound zinc ions are uniformly released by all TFIIIA molecules in the presence of PAR.

Further support to this interpretation...
FIG. 8. 5 S DNA binding and transcriptional activity of TFIIIA species with different zinc stoichiometries. A, 5 S DNA binding. A 450-bp radioactively labeled DNA fragment (1 ng) containing the yeast 5 S rRNA gene was incubated with increasing amounts (2.5, 5, 12.5, and 25 ng) of TFIIIA species containing 9 (lanes 2–5), 6 (lanes 6–9), or 3 (lanes 10–13) zinc eq. The DNA fragment with no added TFIIIA was run in lane 1. DNA-protein complexes were resolved on a 4% non-denaturing polyacrylamide gel and visualized by autoradiography; the positions of free (f) and bound (B) fragments are indicated. B, transcription analysis. Increasing amounts (2, 5, 8, 12, and 16 ng) of zinc-saturated TFIIIA (lanes 2–6), 6 Zn\(^{2+}\)-liganded TFIIIA (lanes 8–12), and 3 Zn\(^{2+}\)-liganded TFIIIA (lanes 14–18) were added to a partially reconstituted transcription system (rTBP, rTFIIB70, fraction B\(^c\), and a TFIIIC/RNA polymerase III fraction) programmed with the 5 S rRNA gene. Control reactions were run in the absence of exogenously added TFIIIA (lanes 1, 7, and 13). The position of transcripts (5 S RNA) after resolution on a polyacrylamide gel is shown; all the lanes shown come from the same exposure of a single gel.

**Table I**

Dissociation constants for zinc-saturated and 6 Zn\(^{2+}\)-liganded TFIIIA-5 S DNA complexes

| TFIIIA species    | \(K_d\) \(\pm\) S.D. | \(N\) | \(n\) |
|-------------------|----------------------|------|------|
| TFIIIA-9Zn\(^{2+}\) | 0.06797 ± 0.02135 | 5    | 57   |
| TFIIIA-6Zn\(^{2+}\)  | 0.06076 ± 0.01896 | 3    | 39   |

was provided by the densitometric analysis of limited proteolysis experiments as the one reported in Fig. 7. Based on the complete loss of detectable polypeptide fragments resulting from trypsin digestion of 3 Zn\(^{2+}\)-liganded TFIIIA (Fig. 7, lane 5), one would in fact predict a reduced recovery of the trypsin-resistant fragment (33.5 kDa) in 6 Zn\(^{2+}\)-liganded as compared with zinc-saturated TFIIIA samples. The extent of such decrease, which would result from degradation of zinc-depleted, inactive TFIIIA molecules, should vary with fractional activity, being most prominent for TFIIIA samples with the highest proportion of inactive molecules (such as the 56% active preparation utilized in the experiment reported in Fig. 7). We found, instead, that regardless of fractional activity, equal amounts of the 33.5-kDa fragment were recovered upon digestion of the 9 Zn\(^{2+}\)- and the 6 Zn\(^{2+}\)-liganded species. Importantly, the total densitometric signal (obtained by summing the p33.5, p30.5, p17, p15, and p11.5 signals) recovered in either case was always the same as the input TFIIIA signal measured in a control undigested sample that was run in parallel (lanes 1, 2, and 5 in Fig. 7, and data not shown). It thus appears that loosely bound zinc ions are not selectively removed from inactive molecules and that a metal stoichiometry of 6 zinc ions/protein molecule is not only sufficient to maintain the structural integrity of TFIIIA but also to preserve its specific 5 S DNA binding and *in vitro* transcription activities. The loss of 3 additional zinc ions, however, results in the formation of a TFIIIA species that is both structurally and functionally impaired.

**Discussion**

Dealing with TFIIIA from the yeast *S. cerevisiae*, this study reports new and, we believe, generally relevant information on the role of zinc ions in determining the structural organization and functional properties of this transcription factor.

Despite the high sequence divergence between yeast TFIIIA and the homologous transcription factor from *Xenopus*, our data indicate that both proteins share a common modular organization. We find, in fact, that the yeast protein is also organized in a two-domain structure consisting of an N-terminal protease-resistant domain of about 34 kDa, with autonomous DNA binding ability, and a smaller C-terminal domain, with a lesser protease resistance, that is responsible for transcription activation. At variance with *Xenopus* TFIIIA, whose DNA-binding domain comprises all the nine zinc fingers, the corresponding domain of the yeast factor includes only eight of the nine fingers, while the ninth finger is located in the activation domain, together with the C-terminal half of the linker region between finger 8 and 9 and the short region C-terminal to the ninth finger. As revealed by limited proteolysis analysis of the yeast transcription factor, these two domains are connected by a region that is highly sensitive to protease cleavage and thus presumably flexible and poorly structured. Interestingly, both trypsin and V8 protease cleave within, or very close to, a cluster of basic residues (RKRRK) that resembles the basic region (KRKLR) interposed between the DNA binding and the activation domains of *Xenopus* TFIIIA (25). In the *Xenopus* factor, however, this basic region lies C-terminal to the ninth finger, a few residues upstream of the activation domain. In yeast TFIIIA, the boundary between the two domains (centered around amino acid residues 325–328) lies about 20 residues upstream with respect to a stretch of hydrophobic amino acids that has recently been shown to play a key role in the assembly of a productive transcription complex (31). The linker region between the eighth and the ninth finger, which represents the site of highest sequence divergence between the yeast and the amphibian transcription factors, thus appears to be a critical determinant of not only the functional but also the structural integrity of yeast TFIIIA. In fact, *in vitro* transcription activity...
was not reconstituted by partially proteolyzed TFIIIA samples, even if they contained both the C-terminal activation domain (p15/p11.5) and the DNA-binding domain (p33.5).

The domain organization of the yeast transcription factor is not stably maintained in an EDTA-treated form of TFIIIA only containing three zinc-coordinated fingers. Interestingly, despite the profound structural alterations exhibited by this 3 Zn\(^{2+}\)-liganded TFIIIA species, including a strong tendency toward aggregation, a native domain organization was recovered following zinc re-supplementation (Fig. 1). Therefore, zinc coordination is not only required to maintain a correct structural organization, but it also promotes the de novo acquisition of such structure starting from a largely disorganized polypeptide chain.

Based on the successful reconstitution and controlled metal depletion of 9 Zn\(^{2+}\)-liganded TFIIIA, the remaining part of our work focused on the comparative characterization of TFIIIA forms with different zinc stoichiometries. This analysis revealed the existence in the yeast protein of at least three previously unidentified classes of zinc-binding sites. The most tightly associated are the 3 zinc ions that remain bound after EDTA treatment, which, as noted above, are not sufficient to maintain TFIIIA integrity. The 3 zinc ions that can be extracted by EDTA, but are not released in the presence of PAR only, identify metal-binding sites of intermediate affinity. The third class of metal coordination sites (estimated \(K_d\) higher than \(10^{-7}\) m) accounts for the 3 zinc ions that are readily released just upon incubation with PAR. The presence of three weak metal-binding sites is consistent with the "spontaneous" loss of 2 zinc eq during protein purification (with the concomitant oxidation of 4 cysteine residues) and with the fact that the resulting 7 Zn\(^{2+}\)-liganded species slowly released to PAR only 1 additional zinc ion. This indicates that the 2 zinc eq lost in the course of protein purification correspond to the 2 zinc ions that are rapidly released from metal-saturated TFIIIA in the presence of PAR. This observation, along with the strongly biphasic kinetics of loosely bound zinc release, points to the existence of PAR. This observation, along with the strongly biphasic kinetics of loosely bound zinc release, points to the existence in yeast of at least three classes of zinc-binding sites with very different metal affinities. The presence of 3 nonessential zinc ions considerably limits, at least in yeast, the effectiveness of previously proposed regulators of transcription factor activity,2 as the simultaneous binding of 5 S DNA by all nine fingers involves a substantial energetic cost (33). The apparent redundancy of 3 zinc ions in yeast TFIIIA, as revealed by the present analysis of an unmodified, full-length protein, is thus probably best explained by the remarkably high degree of structural and functional flexibility built into this complex transcription factor. A similar case of structural and functional heterogeneity among zinc fingers has recently been reported for human metal-response element-binding transcription factor-1, where a subset of loosely bound zinc ions can be removed without effect on the structure and DNA binding ability (56).

Finally, it may be useful to consider the possible physiological implications of the existence in yeast TFIIIA of three classes of zinc-binding sites with very different metal affinities. The presence of 3 nonessential zinc ions considerably limits, at least in yeast, the effectiveness of previously proposed regulatory mechanisms based on the zinc-dependent modulation of TFIIIA activity in response to changes in zinc availability (57). Far from being key regulators of transcription factor activity, the three loose metal-binding fingers of yeast TFIIIA may instead be viewed as an intramolecular zinc reservoir that can allow the synthesis of an essential component of the translation
machinery, the 5 S rRNA, even under temporary conditions of severe zinc shortage.

Acknowledgments—We are grateful to Gian Luigi Rossi for helpful discussions and support, to Tony Wei for plasmid pUC9-5 S, and to Owen Rowland and Jacqueline Segall for communicating results prior to publication. We also thank Alessio Peracchi and Riccardo Percudani for their critical reading of the manuscript.

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Domain Organization and Functional Properties of Yeast Transcription Factor IIIA Species with Different Zinc Stoichiometries
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J. Biol. Chem. 1999, 274:2539-2548.
doi: 10.1074/jbc.274.4.2539

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