

## A Position-Dependent Transcription-Activating Domain in TFIIIA

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**Transcription of the *Xenopus* 5S RNA gene by RNA polymerase III requires the gene-specific factor TFIIIA. To identify domains within TFIIIA that are essential for transcriptional activation, we have expressed C-terminal deletion, substitution, and insertion mutants of TFIIIA in bacteria as fusions with maltose-binding protein (MBP). The MBP-TFIIIA fusion protein specifically binds to the 5S RNA gene internal control region and complements transcription in a TFIIIA-depleted oocyte nuclear extract. Random, cassette-mediated mutagenesis of the carboxyl region of TFIIIA, which is not required for promoter binding, has defined a 14-amino-acid region that is critical for transcriptional activation. In contrast to activators of RNA polymerase II, the activity of the TFIIIA activation domain is strikingly sensitive to its position relative to the DNA-binding domain. When the eight amino acids that separate the transcription-activating domain from the last zinc finger are deleted, transcriptional activity is lost. Surprisingly, diverse amino acids can replace these eight amino acids with restoration of full transcriptional activity, suggesting that the length and not the sequence of this region is important. Insertion of amino acids between the zinc finger region and the transcription-activating domain causes a reduction in transcription proportional to the number of amino acids introduced. We propose that to function, the transcription-activating domain of TFIIIA must be correctly positioned at a minimum distance from the DNA-binding domain.**

Initiation of transcription by eukaryotic RNA polymerases requires the formation of specific protein complexes at gene promoter elements. RNA polymerase III transcribes a number of small genes, such as 5S RNA and tRNA genes, that have promoters within the transcription unit (reviewed in reference 20). Many of the transcription factors required for RNA polymerase III transcription have been identified, and their order of assembly on 5S RNA genes has been clearly established, particularly for *Xenopus laevis* and *Saccharomyces cerevisiae*, yet little is known of the actual mechanism of transcriptional activation (3, 31, 43). Although there are undoubtedly species-specific differences, some generalizations can be made regarding the assembly and properties of transcription complexes formed on 5S RNA genes. Assembly of a transcription complex on the 5S RNA gene is initiated by sequence-specific binding of the zinc finger protein TFIIIA to the internal control region (ICR) (15, 21, 34). Components of TFIIIC bind next and stabilize the binding of TFIIIA to the gene, committing the template for transcription. TFIIIC requires specific DNA base pairs within A- and C-box elements of the ICR (33). However, it does not bind to the ICR of 5S RNA genes in the absence of TFIIIA (7, 44). In contrast, independent sequence-specific DNA binding by factors in the TFIIIC fraction is observed at A- and B-box elements of tRNA genes (6, 16, 45). Assembly of the preinitiation complex is completed by TFIIIB. One component of TFIIIB is the TATA box-binding protein, which is required for transcription by RNA polymerases I, II, and III (9, 29, 32, 46, 49). TFIIIB specifically interacts with an upstream DNA-binding site only after assembly I into the transcription complex (2, 27, 30). The activation mechanism for DNA binding by TFIIIB is unknown but presumably results from protein-protein interactions. The

completed complex is highly stable to multiple rounds of transcription by RNA polymerase III, both in vivo and in vitro (5, 10).

5S RNA gene expression in *Xenopus* spp., unlike that in *S. cerevisiae*, is developmentally regulated at the transcriptional level (52). Fundamental differences may exist in the functions of individual proteins within the transcription complexes assembled on yeast and *Xenopus* class III genes. In yeast cells, TFIIIA and TFIIIC have been shown to function primarily as assembly factors. DNase I protection assays show that heparin can strip TFIIIA and TFIIIC from the complete transcription complex without disrupting accurate transcription initiation (28). It remains to be established whether *Xenopus* TFIIIA is required solely for transcription complex assembly. However, TFIIIA probably remains in the transcription complex after assembly. DNase I protection assays of *Xenopus* 5S RNA genes that are being actively transcribed in *Xenopus* oocyte nuclear extracts at in vivo rates show an extended region of protection compared with TFIIIA alone. During assembly of the complex, a characteristic TFIIIA footprint is produced, followed by extension toward the 5' end of the gene (53, 54). In isolated *Xenopus* erythrocyte chromatin, the somatic 5S RNA genes require only RNA polymerase III for activation of transcription, suggesting that functional transcription complexes remain associated with the genes even in a cell that is transcriptionally dormant (10). The continued presence of TFIIIA in these complexes is supported by low-resolution micrococcal nuclease digestion of *Xenopus* erythrocyte chromatin, which shows protection of a single cleavage site characteristic of the presence of TFIIIA (8).

TFIIIA is the only component of the 5S RNA gene transcription complex that has been cloned from *Xenopus* spp. (21). *Xenopus* and yeast TFIIAs show little homology at the primary sequence level (1). Both proteins have nine zinc fingers, but the spacing of the zinc fingers is different.

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These differences may be related to transcriptional properties or to the requirement of particular zinc fingers of *Xenopus* TFIIIA to bind both 5S DNA and 5S RNA (11, 47). The carboxyl-terminal region of *Xenopus* TFIIIA (the 69 amino acids following zinc finger 9) plays an important function in activation of transcription from the initiation complex. Deletion of the C-terminal region eliminates the transcriptional function of TFIIIA but does not affect DNA binding by the residual nine-zinc-finger fragment (11, 48). The mechanism of transcription activation by the C terminus of TFIIIA is unknown. It may function indirectly, by positioning TFIIIB or TFIIIC during assembly of the complex, or directly by interacting with RNA polymerase III or another transcription factor. A direct interaction of this domain with TFIIIC is suggested by reduced stability of transcription complexes formed with a 34-kD proteolytic fragment of TFIIIA that lacks part of the C-terminal region (25). The TFIIIA C-terminal amino acid sequence shows no homology with sequences of the known RNA polymerase II transcriptional activators, such as a high proportion of acidic, glutamine, or proline residues (35). Carboxyl-terminal zinc fingers may also be required for transcriptional activation in addition to DNA binding. Disruptions of single zinc fingers in the C-terminal portion of the zinc finger domain, made by mutation of a single histidine to asparagine, result in reduced transcription without changing the affinity of TFIIIA for the ICR (13).

As a first step toward understanding the mechanism of transcriptional activation by TFIIIA, we have defined precisely the C-terminal amino acids required for transcription. We have constructed deletion, substitution, and insertion mutants in the TFIIIA cDNA and expressed these constructs in *Escherichia coli* as fusions with maltose-binding protein (MBP). Transcriptional activity of the purified mutant proteins was measured by complementation of an oocyte nuclear extract immunodepleted of endogenous TFIIIA. We identify a short region of 14 to 18 amino acids within the C-terminal region of TFIIIA that is indispensable for transcriptional activation. The activity of this domain is sensitive to small changes in its location in the protein relative to the zinc finger domain.

## MATERIALS AND METHODS

**Synthetic oligonucleotides.** The following synthetic oligonucleotides were used (all are shown 5' to 3'; underlined nucleotides in BRranP were synthesized with 76% of the parental base and 8% of each other base; "N" indicates an equal mixture of dA, dC, dG, and dT at that position): P673, d(GGG ATC ATC AGA AAA CTC ACG); PΔ40RP, d(TGG AAG CTT AGG GGG GTA TGT ATC CAG TGA G); PΔ45RP, d(TGG AAG CTT AAG TGA GGC GAG AGG CCA GGC T); PΔ50RP, d(TGG AAG CTT ACA GGC TTC TCT TTG GGC GAG G); PΔ55RP, d(TGG AAG CTT AGC GAG GGC ATT TCT CCT TCA G); RP1619, d(GCG GAT AAC AAT TTC ACA CAG); ZF9RP, d(TGC AGC CGG CAT GTA CAA CTG AAT GTC TTT C); DSMP, d(ACG TGC CGG CGA GAA GAG GAA GCT GAA GGA G); BRIP, d(ACG TGC CGG CTT TCT CCT TCA GCT TCC TC); BRDP, d(ACG TGC CGG CTG CCC TCG CCT AAA GAG AAG C); FDIP, d(ACG TGC CGG CGC CTC TCG CCT CAC TGG ATA C); FDIIP, d(ACG TGC CGG CGG ATA CAT ACC CCC CAA GAG C); BRranP, d(A CGT GCC GGC GAG AAG AGG AAG CTG AAG GAG AAA TGC CCT CGC CCA AAG AGA AGC CTG GCC); CTRanFP1, d(CGC CTC ACC GGT NNC ANN CNN CNN

ANN AGC AAA GAA AAA AAT GCA TCC GTT TCG); CTRanRP1, d(GGG GTA TGT AAC CGG TGA GGC GAG AGG); CTRanFP2, d(C CCA AAG AGA TCC TTG GCC NNC CNN ANN GNN TAC ATA CCC CCC AAG AGC AAA GAA); CTRanRP2, d(GAG GCG AGA GGC CAA GGA TCT CTT TGG GCG); CTRanFP3, d(GAG AAA TGC CCT CGG CCG ANN ANN ANN CNN GNN TCT CGC CTC ACT GGA TAC ATA CCC); and CTRanRP3, d(CAG GCT TCT CTT CGG CCG AGG GCA TTT CTC).

**Plasmid constructs.** (i) **pMTF.** The cDNA encoding *Xenopus laevis* TFIIIA was subcloned into the MBP expression vector pMALc2 (New England Biolabs, Inc.) as follows. Oligonucleotides 5'-ATGGGTGAAAAAGCTCTG and 5'-CCGGCAGAGCTTTTTACCCAT were hybridized to form a double-stranded adapter and ligated to the *Cfr*10I-BamHI fragment of the TFIIIA cDNA from pSPTF15 (48). This fragment was ligated with an *Xmn*I-BamHI fragment from pMALc2 and used to transform *Escherichia coli* DH5α competent cells.

(ii) **Carboxyl-terminal deletion constructs.** pMTF1-304, pMTF1-299, pMTF1-294, and pMTF1-289, encoding C-terminal truncations of MBP-TFIIIA fusions (mTFIIIA) indicated by the number of amino acids remaining, were generated by ligation of polymerase chain reaction (PCR)-generated DNA fragments into pMTF. Template DNA (3 fmol of pSPTF15) was mixed with 100 pmol of a forward primer, p673, located 27 nucleotides 5' of the unique *Xho*I site, and 100 pmol of the appropriate reverse primers, PΔ40RP, PΔ45RP, PΔ50RP, and PΔ55RP, which contain a *Hind*III site and a TAA stop codon. PCR amplification was carried out for 25 cycles, using *Taq* DNA polymerase (GeneAmp kit; Perkin Elmer) (41). The length of each PCR product was confirmed by agarose gel electrophoresis in TBE (89 mM Tris, 89 mM borate, 1 mM EDTA [pH 8.3]). PCR mixtures were extracted with chloroform and precipitated with ethanol. The PCR-generated DNA fragment was digested with *Xho*I and *Hind*III and ligated into the 7,278-bp *Xho*I-*Hind*III fragment from pMTF. The nucleotide sequence of the PCR-generated fragment was confirmed by the dideoxy-chain termination method.

(iii) **pMSTF and internal deletion constructs.** pMSTF encodes an MBP fusion of TFIIIA in which a double amino acid substitution (D277A, P278G) has been made at the end of zinc finger 9 in order to introduce an *Ngo*MI site for further mutagenesis; the encoded mutants are designated by the prefix "ms." Template DNA (pSPTF15) was amplified in two PCRs. In the first reaction, the forward primer was DSMP, a 31-base oligonucleotide at the end of zinc finger 9 in which the sequence GAT CCA (encoding amino acids D and P) has been changed to the *Ngo*MI recognition sequence GCC GGC (encoding amino acids A and G), and the reverse primer was RP1619, which is complementary to a vector sequence 3' to the TFIIIA cDNA. In the second reaction, the reverse primer was ZF9RP, the complement of DSMP, which contains an *Ngo*MI site near the end of zinc finger 9, and P673 was the forward primer. The two PCR products were digested with *Ngo*MI and ligated. The ligation mixture was digested with *Xho*I and *Hind*III, and the recombinant fragment containing an *Ngo*MI site was purified from an agarose gel and ligated into the 7,278-bp *Xho*I-*Hind*III fragment of pMTF.

Internal deletion constructs pMSTFΔ279-286, pMSTFΔ279-291, and pMSTFΔ279-295 were constructed in a similar manner. In the first PCR, forward primers complementary to the 3' limit of the desired deletion (BRDP, FDIP, and FDIIP, respectively) were used to generate an *Ngo*MI-*Hind*III frag-

ment. This fragment was ligated to the *XhoI*-*NgoMI* fragment from the second PCR, using primers P673 and ZF9RP, and subcloned into pMSTF as described above.

(iv) **pxMTFfranbr.** In our construction scheme for random replacement of nucleotides encoding the basic region, it was first necessary to eliminate an *NgoMI* site in the vector. pMSTF was digested with *DraIII* and *NgoMI*, ends were filled with Klenow fragment and deoxynucleotides, and the DNA was self-ligated to form pxMTF. The 845-bp *XhoI*-*HindIII* fragment in pxMTF was substituted by the 845-bp *XhoI*-*HindIII* fragment from pMSTF to form pxMSTF. Randomized stretches of nucleotides were introduced via a PCR primer. PCR amplification on pSPTF15 was carried out with BRranP as the forward primer and RP1619 as reverse primer. BRranP is a 61-base oligonucleotide mixture in which there is a 24-base random segment and a 5' *NgoMI* site. The oligonucleotide was synthesized at each of the degenerate positions with 76% of the parental base and 8% of each other base to decrease the sequence complexity of the final products. The PCR product, which is a mixture of sequences of defined size, was subcloned into *NgoMI*- and *HindIII*-digested pxMSTF.

(v) **pxMTFfran291-295, pxMTFfran296-300, and pxMTFfran301-305.** Randomization of blocks of 15 nucleotides was done in pxMTF to introduce specific segments of random amino acids in mTFIIIA. As for the construction of pxMTFfranbr, random segments which covered the appropriate regions (CTRanFP1 for amino acids 301 to 305, CTRanFP2 for amino acids 296 to 300, and CTRanFP3 for amino acids 291 to 295) and contained a 5' restriction endonuclease site that did not change the encoded amino acids (*BsrFI* for ran301-305, *StyI* for ran296-300, and *EagI* for ran291-295) were introduced by PCR primers. As in the construction of pMSTF, two PCRs were carried out with pSPTF15 as the template. The first used the CTRanFP primers as forward primers and RP1619 as the reverse primer. The second reaction used P673 as forward primers and reverse primers complementary to the CTRanFP primers, CTRanRP1, -2, and -3. PCR products from both reactions for each mutated site were digested at the introduced restriction enzyme site and ligated to each other. The ligation products were digested with *HindIII* and *XhoI* and subcloned into the *XhoI*-*HindIII* fragment of pxMSTF.

(vi) **Basic region insertion constructs.** Insertion mutants (pxMSTFBRI-1, -2, and -3) were constructed by insertion of a 30-bp DNA fragment encoding the basic region of TFIIIA into the *NgoMI* site of pxMSTF. This fragment was prepared by *NgoMI* digestion of a larger PCR fragment that had been made with forward primer P673, reverse primer BRIP, which contains an *NgoMI* site at its 3' end, and pMSTF as the template.

(vii) **Plasmid templates for transcription.** Plasmid pUXbs, containing a single copy of the *Xenopus borealis* somatic-type 5S RNA gene (38), was prepared by subcloning the *HindIII* fragment of pXbs1 into pUC18. Similarly, pUTM10, encoding a single methionyl-tRNA gene (36), was prepared by subcloning the *BamHI*-*EcoRI* fragment of pTM10 into pUC18.

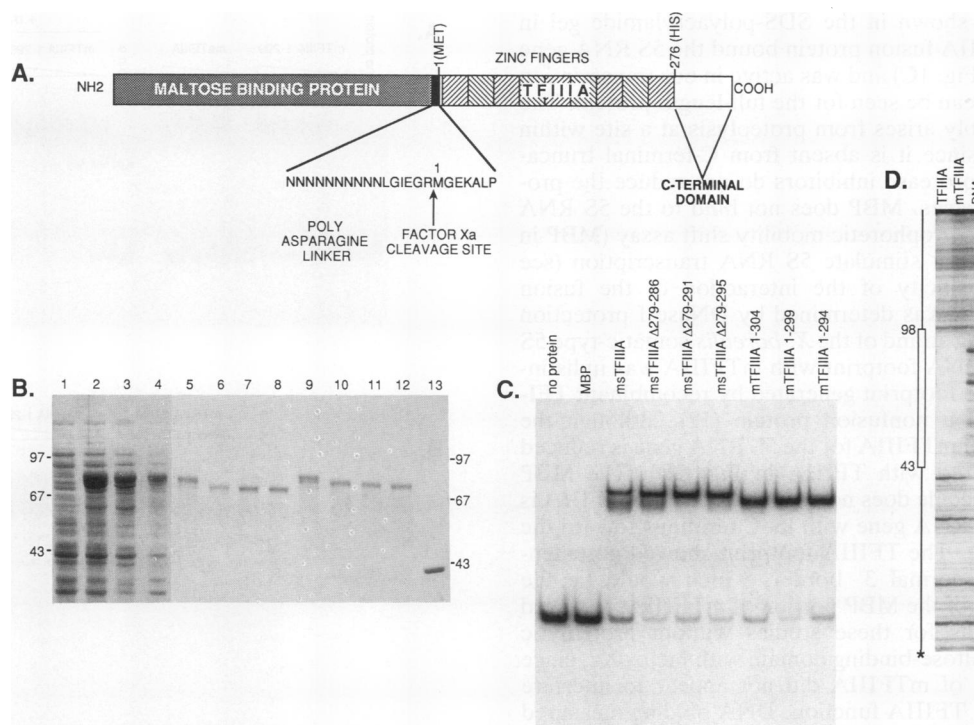
**Expression and purification of mTFIIIA proteins.** Two milliliters of rich broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 2 g of glucose per liter [pH 7.0]) containing 100  $\mu$ g of ampicillin per ml and 10  $\mu$ M ZnSO<sub>4</sub> was inoculated with a single colony of *E. coli* DH5 $\alpha$  containing each plasmid construct and shaken overnight at 37°C. Cells were sedimented by centrifugation at 1,500  $\times$  g for 5 min and resuspended in 2.0 ml of fresh medium; 0.5 ml was used to

inoculate 50 ml of rich broth. Cells were shaken at 37°C until the A<sub>600</sub> of the culture reached 0.5. Expression of fusion protein was induced by addition of 0.5 mM isopropylthiogalactopyranoside (IPTG), and the cells were shaken for 2 h at 37°C. Cells were harvested by centrifugation at 10,000  $\times$  g for 10 min at 4°C, resuspended in 2 ml of 1 $\times$  NCB buffer (250 mM NaCl, 1 mM dithiothreitol [DTT], 20  $\mu$ M ZnSO<sub>4</sub>, 1 mM benzamidine, 20 mM Tris-HCl [pH 7.5]), and lysed by three freeze-thaw cycles and sonication. The cell lysate was clarified by centrifugation at 10,000  $\times$  g for 15 min at 4°C. All of the fusion proteins that we have expressed with one exception, mTFIIIA 1-304, are in the supernatant fraction of the centrifuged cell lysate. Renaturation of fusion protein was achieved by slowly mixing 0.2 ml of supernatant with 0.8 ml of 6.25 M urea in NCB and incubating the mixture for 16 h at 2°C. Urea was removed by dialysis in two steps. The mixture was dialyzed against 50 volumes of 2.5 M urea in NCB for 3 h at 2°C and then dialyzed against 50 volumes of NCB for 3 h at 2°C. Protein in the pellet fraction was extracted by first resuspending the pellet with 0.2 ml of NCB and then adding 0.8 ml of 6.25 M urea and mixing the suspension overnight at 2°C. The extract was cleared by centrifugation at 10,000  $\times$  g for 15 min at 4°C, and urea was removed from the supernatant by dialysis as described above. MBP fusion protein was bound batchwise to 0.1 ml of packed amylose-Sepharose beads by mixing overnight at 2°C. Beads were washed six times with 1 ml of NCB buffer and eluted with 0.2 ml of 20 mM maltose-10% glycerol in NCB. The concentration of eluted protein was determined by the Bradford assay (5a), using bovine serum albumin as a standard.

**Transcription reactions.** Oocyte nuclear extract was prepared from manually isolated germinal vesicles (approximately two to three per microliter) as described previously (4). TFIIIA in the extract was depleted with an anti-TFIIIA antiserum. Fifty microliters of extract was incubated with 5  $\mu$ l of antiserum in J buffer (70 mM NH<sub>4</sub>Cl, 7 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 2 mM DTT, 8% glycerol, 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.4]) containing 0.1% Nonidet P-40 (NP-40) for 30 to 60 min on ice. This mixture was transferred to 30  $\mu$ l of immobilized protein A beads that were prewashed with J buffer and incubated with occasional mixing for 30 min on ice. The supernatant was used immediately for transcription or quick-frozen in dry ice-ethanol and stored at -80°C without significant loss of activity.

Transcription reactions (12  $\mu$ l) were carried out in J buffer with 6  $\mu$ l of depleted oocyte nuclear extract, 12 to 120 nM recombinant fusion protein, 0.2 mM each ATP, GTP, and UTP, 0.02 mM CTP, 5  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]CTP, 4.7 nM DNA (1.4 nM pUXbs and 3.3 nM pUTM10), 0.1% NP-40, and 4 U of RNase inhibitor. Reactions were started by the addition of nucleotides and incubated for 60 min at room temperature (21°C). Reactions were stopped with 0.1 ml of 0.3 M sodium acetate (pH 5.2)-0.1% sodium dodecyl sulfate (SDS)-10 mM EDTA-80  $\mu$ g of yeast tRNA carrier per ml and extracted with phenol, phenol-chloroform (1:1, vol/vol), and then chloroform, and RNA was precipitated with 2.5 volumes of ethanol. Pelleted RNA was dissolved in 90% formamide, and labeled transcripts were separated by electrophoresis through 8% polyacrylamide gels (19:1 acrylamide/bisacrylamide) containing 45 mM Tris-borate (pH 8.3), 1 mM EDTA, and 7 M urea. Gels were dried under vacuum and exposed to Kodak XAR-5 autoradiographic film.

**Electrophoretic mobility shift assay.** A 3'-<sup>32</sup>P-labeled 66-bp DNA fragment from the *X. borealis* 5S RNA gene ICR was



**FIG. 1.** Structures and properties of MRP-TFIIIA (mTFIIIA) constructs. (A) Primary structure of the mTFIIIA constructs expressed from plasmid pMTF. Amino acid numbering refers to positions in *X. laevis* TFIIIA and begins at the first methionine of TFIIIA. Zinc fingers are represented by hatched boxes. Amino acids are shown in single-letter code. (B) Purification of mTFIIIA mutants. Iodoacetylated proteins were separated by electrophoresis on a 8% polyacrylamide gel in the presence of SDS and stained with Coomassie blue R250. Shown are results for uninduced *E. coli* DH5 $\alpha$  (lane 1), IPTG-induced cells (lane 2), soluble fraction of cell lysate (lane 3), insoluble fraction of cell lysate (lane 4); mTFIIIA (lane 5), C-terminal deletion proteins mTFIIIA 1-304, 1-299, and 1-294 (lanes 6 to 8), insertion mutant BRI-3 (lane 9), internal deletion mutants msTFIIIA  $\Delta$ 279-286,  $\Delta$ 279-291, and  $\Delta$ 279-295 (lanes 10 to 12), and MBP (lane 13). Positions of molecular weight markers (phosphorylase *a* [97,000], bovine serum albumin [67,000], and ovalbumin [43,000]) are indicated in thousands. (C) Binding of mTFIIIA to 5S RNA ICR DNA. The indicated fusion proteins (50 nM) were incubated with a  $^{32}$ P-labeled 66-bp fragment from the *X. borealis* 5S RNA gene (3 nM), and DNA-protein complexes were resolved by electrophoresis through a 5% nondenaturing polyacrylamide gel. (D) DNase I protection of mTFIIIA and TFIIIA binding to the *X. borealis* 5S RNA gene. Proteins (10 nM recombinant TFIIIA and 60 nM mTFIIIA) and were incubated with a 238-bp DNA fragment from pXbs201 (1.2 nM) uniquely labeled at the 3' end of the coding strand and treated with DNase I. Fragments were separated by denaturing polyacrylamide electrophoresis. Lane DNA is a digestion of DNA in the absence of protein. The position of the ICR of the *X. borealis* 5S RNA gene in this DNA fragment is indicated by the open box at the left. The asterisk indicates the position of the radiolabel.

incubated with recombinant fusion protein in 70 mM KCl–10  $\mu$ M ZnSO<sub>4</sub>–2.5 mM MgCl<sub>2</sub>–0.1% NP-40–5 mM DTT–5% glycerol–20 mM Tris-HCl (pH 7.5)–10  $\mu$ g of poly(dI-dC) per ml (total volume, 10  $\mu$ l) for 15 min at room temperature. The mixture was loaded directly onto a 1-mm-thick 5% polyacrylamide gel (29:1 acrylamide/bisacrylamide) in 0.5 $\times$  TBE buffer. Electrophoresis was carried out for 60 to 90 min at 150 V. Gels were dried under vacuum and exposed to Kodak XAR-5 autoradiographic film.

**DNase I protection assay.** DNA footprinting reactions were carried out on the 238-bp *Bam*HI-*Hind*III fragment of pXbs201 that was uniquely end labeled at the 3' end of the coding strand with Klenow fragment and [ $\alpha$ - $^{32}$ P]dCTP. Recombinant TFIIIA (12) (10 nM) or mTFIIIA (60 nM) was incubated with DNA (1.2 nM) in 70 mM KCl–10  $\mu$ M ZnSO<sub>4</sub>–2.5 mM MgCl<sub>2</sub>–0.1% NP-40–5 mM DTT–5% glycerol–20 mM Tris-HCl (pH 7.5)–10  $\mu$ g of poly(dI-dC) per ml for 15 min at room temperature. Ten nanograms of DNase I (Boehringer Mannheim) was added, and incubation continued for 90 s. The reaction was stopped with 0.05 ml of 0.3 M sodium acetate (pH 5.2)–0.1% SDS–10 mM EDTA–50  $\mu$ g of glycogen carrier per ml and extracted with phenol-chloroform

(1:1, vol/vol), and DNA was precipitated with ethanol. DNA fragments were resuspended with 90% formamide and separated on 8% polyacrylamide gels containing 8 M urea.

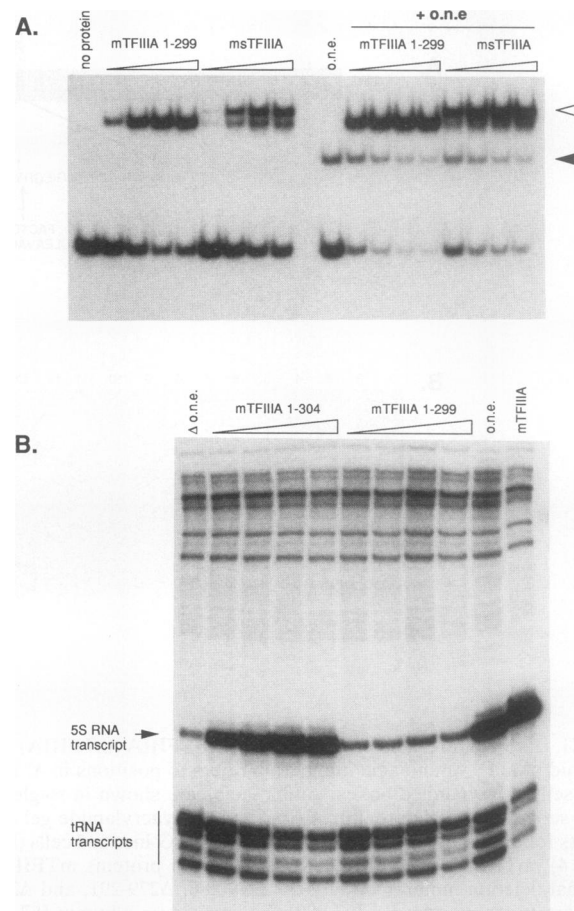
## RESULTS

**Expression of TFIIIA as an MBP fusion.** The cDNA encoding *X. laevis* TFIIIA was subcloned into the expression vector pMALc2, and TFIIIA with an N-terminal fusion of MBP (22) was expressed in *E. coli*. Primary structural features of the fusion protein are summarized in Fig. 1A. We chose this expression system because the 82-kD fusion protein (mTFIIIA) is soluble in the bacterial cytoplasm and is purified easily to near homogeneity by amylose column chromatography (Fig. 1B). DNA affinity of the fusion protein was improved by renaturing the protein by dialysis from 5 M urea in the presence of zinc sulfate and DTT. This renaturation step can be performed prior to amylose chromatography without reducing the affinity of MBP for amylose. Other systems for bacterial expression of TFIIIA have used similar measures to renature the polypeptide (12, 37). A purification profile and purified fusion proteins from the different classes

of mutations are shown in the SDS-polyacrylamide gel in Fig. 1B. The TFIIIA fusion protein bound the 5S RNA gene ICR (mTFIIIA; Fig. 1C) and was active in our transcription assay. A doublet can be seen for the full-length protein. The lower band possibly arises from proteolysis at a site within the C terminus, since it is absent from C-terminal truncations. However, protease inhibitors do not reduce the proportion of this species. MBP does not bind to the 5S RNA gene ICR in an electrophoretic mobility shift assay (MBP in Fig. 1C), nor does it stimulate 5S RNA transcription (see Fig. 6). The specificity of the interaction of the fusion proteins with DNA was determined by DNase I protection assay on the coding strand of the *X. borealis* somatic-type 5S RNA gene. The DNA footprint with mTFIIIA was indistinguishable from the footprint generated by recombinant TFIIIA expressed as a nonfusion protein (12), although the apparent affinity of mTFIIIA for the 5S RNA gene is reduced (mTFIIIA compared with TFIIIA in Fig. 1D). The MBP portion of the molecule does not protect the DNA. TFIIIA is aligned on the 5S RNA gene with its C terminus toward the 5' end of the gene. The TFIIIA footprint showed no extension beyond the normal 3' border, which would be the expected position of the MBP portion of mTFIIIA. We used the fusion proteins for these studies without proteolytic removal of the maltose-binding domain with factor Xa, since the MBP portion of mTFIIIA did not appear to interfere substantially with TFIIIA function. DNA binding measured by titration of protein in a mobility shift assay is reduced by approximately 25% (data not shown).

**C-terminal deletions of mTFIIIA define a five-amino-acid domain essential for transcriptional activation of 5S RNA genes.** To define the transcription activating domain, we constructed a series of five-amino-acid C-terminal deletions from position 313. Our starting point was determined by previous studies that showed a 31-amino-acid C-terminal truncation of TFIIIA (TFIIIA 1-313) was able to support DNA binding and transcription (48). C-terminal deletions between amino acids 304 and 294 were able to bind a radiolabeled 5S RNA gene ICR oligonucleotide in an electrophoretic mobility shift assay (mTFIIIA 1-304, 1-299, and 1-294; Fig. 1C). The binding of the truncated proteins was enhanced by either a complete oocyte nuclear extract (mTFIIIA 1-299; Fig. 2A) or an oocyte nuclear extract immunodepleted of endogenous TFIIIA (data not shown). This enhancement may result from stabilization of TFIIIA binding by TFIIIC in the extract, which does not cause a change in the mobility of TFIIIA (25, 50). The molecular basis for stabilization by TFIIIC without a change in DNA mobility is unknown. TFIIIC could act transiently to stabilize TFIIIA binding through a conformational change, or the DNA-TFIIIA-TFIIIC complex may be unstable in our electrophoresis conditions.

Transcriptional activity of the mutant proteins was measured by complementing a TFIIIA-depleted oocyte nuclear extract (Fig. 2B). Two plasmids were added to extracts supplemented with fusion protein to monitor transcription. Plasmid pUXbs contains a single copy of the *X. borealis* somatic-type 5S RNA gene in pUC18, and plasmid pUTM10 contains a single copy of the *X. laevis* methionyl-tRNA gene. The tRNA gene serves as an internal control for the specificity of depletion of the oocyte nuclear extract with anti-TFIIIA immunoglobulin G, since its transcription is independent of TFIIIA. The tRNA gene, however, shares a requirement with the 5S RNA gene for TFIIIC, TFIIIB, and RNA polymerase III; therefore, its transcription relative to the 5S RNA gene provides a measure of the competition for



**FIG. 2.** A five-amino-acid deletion in the C terminus of mTFIIIA eliminates transcriptional activity without changing DNA binding. (A) DNA-binding enhancement by oocyte nuclear extract. A 66-bp DNA fragment from the *X. borealis* gene (3 nM) was incubated with increasing amounts of the indicated fusion protein with and without oocyte nuclear extract (o.n.e.), and protein-DNA complexes were resolved by nondenaturing electrophoresis. Triangles at the top represent increasing amounts (25, 50, 75, and 100 nM) of fusion protein. The open arrowhead indicates the position of fusion protein-DNA complexes; the closed arrowhead indicates the position of DNA complexed with TFIIIA in the oocyte nuclear extract. (B) Transcriptional activity of C-terminal deletion proteins. Immunodepleted oocyte nuclear extract was complemented with purified fusion proteins and used to transcribe a mixture of *X. borealis* somatic-type 5S RNA and *X. laevis* tRNA<sup>Met</sup> genes. Each triangle spanning four lanes represents increasing amounts (12, 25, 50, and 75 nM) of the indicated purified fusion protein. Fusion protein was not added to depleted oocyte nuclear extract in the transcription reaction in lane ( $\Delta$  o.n.e.). Reactions were complemented with 60 nM full-length fusion protein in lane mTFIIIA. Transcription with complete oocyte nuclear extract is shown in lane o.n.e. Transcripts were labeled by inclusion of [ $\alpha$ -<sup>32</sup>P]CTP in the reaction and separated by denaturing polyacrylamide gel electrophoresis.

these factors between mTFIIIA-5S DNA complexes and tDNA.

mTFIIIA has transcriptional activity comparable to that of recombinant TFIIIA and oocyte TFIIIA. A 0.12-pmol amount of mTFIIIA supported transcription to a level corresponding to 74% of that supported by 1.5  $\mu$ l of unfractionated oocyte nuclear extract, which contains approximately 0.07 to 0.21 pmol of TFIIIA (50) (Fig. 2, densitometric

comparison of lanes mTFIIIA and o.n.e.). In our reconstituted transcription system, mTFIIIA supported 5S RNA transcription at 70% of the level supported by wild-type TFIIIA when protein concentrations were saturating (data not shown).

We found that the presence of five amino acids between positions 300 and 304 (Tyr-Ile-Pro-Pro-Lys) was essential for transcriptional activation of the 5S RNA gene by TFIIIA. The C-terminal truncated protein TFIIIA 1-304 had transcriptional activity comparable to that the full-length protein, whereas mTFIIIA 1-299 was unable to stimulate 5S RNA transcription above that in depleted oocyte nuclear extract alone (Fig. 2B). The 5S RNA transcript in depleted oocyte nuclear extract (Fig. 2B, lane  $\Delta$ o.n.e.) arises from incomplete depletion of this batch of extract. Transcription from the tRNA gene decreased as more mTFIIIA 1-299 or 1-304 was added, even in the absence of transcription from the 5S RNA gene. This observation suggests mTFIIIA 1-299 retains interactions with a shared factor(s).

The difference in transcriptional activity between mTFIIIA 1-299 and mTFIIIA appears unrelated to differences in DNA-binding affinity. Both proteins were able to bind to the ICR (Fig. 1C), and we expected them to have the same affinity for the 5S RNA gene ICR, since they have the same complement of zinc fingers. To eliminate transcriptional effects related to any small differences in DNA-binding affinity that may exist, we titrated the proteins to saturating transcriptional activity. In addition, DNA binding of the transcriptionally inactive mutant, mTFIIIA 1-299, and mTFIIIA was enhanced by factors in the oocyte nuclear extract. Figure 2A shows the stabilization of fusion protein binding to DNA in the presence and absence of oocyte nuclear extract. The interaction of the fusion protein with the ICR eventually competed for binding by endogenous TFIIIA in the complete extract.

**Definition of a TFIIIA transcriptional activation domain by random mutagenesis.** To determine whether only five amino acids (300 to 304) comprise the transcription-activating domain or whether they are the C-terminal end of a larger domain, we substituted neighboring five-amino-acid segments within the full-length fusion protein with random amino acid cassettes (40). Substitution cassettes, containing 15 nucleotides of the cDNA randomized at two positions in each coding triplet, were individually introduced into the mTFIIIA cDNA at three adjacent sites. Codons were randomized at only two positions to avoid stop codons that would create truncated proteins. In the absence of a selection procedure based on transcriptional activity, we selected clones at random, purified full-length fusion proteins and measured their transcriptional activity by complementing TFIIIA-depleted oocyte nuclear extract. As a positive control for the renaturation procedure during protein purification, a fresh colony of mTFIIIA was prepared together with each set of mutated proteins.

Transcription assays with mTFIIIA containing randomly substituted blocks of 5 amino acids showed that a region of at least 15 amino acids in the C terminus is critical for transcription. The sequences of mutant proteins and their transcriptional activities are compiled in Fig. 3. This region is sensitive to amino acid changes. Strikingly, only 1 of 20 mutants assayed had any transcriptional activity. One inactive mutant had just two amino acid changes (S296A and G300E). The active mutant stimulated transcription to approximately 20% of that with mTFIIIA and was altered at all five amino acid positions between 301 and 305 (Tyr-Ile-Pro-Pro-Lys to Val-Thr-Arg-Leu-Arg; Fig. 3, second line). Of 11

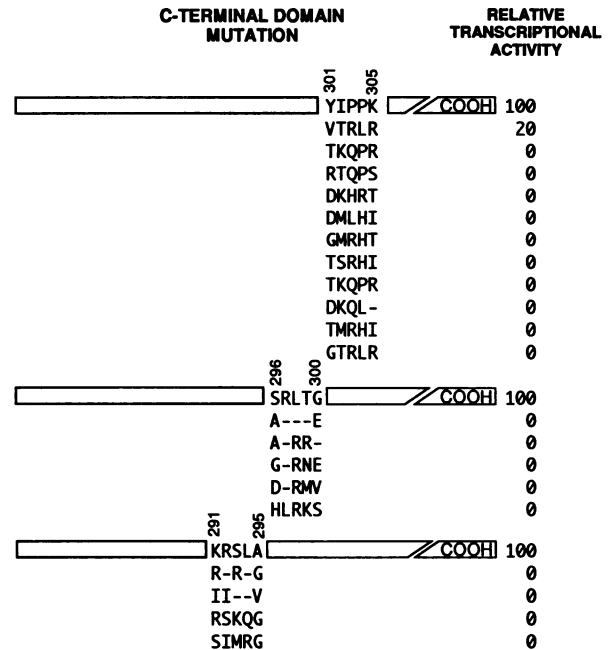


FIG. 3. A 15-amino-acid region in the C terminus of TFIIIA is indispensable for transcription. A summary of random-substitution mutagenesis is shown. Blocks of five amino acids in the C terminus of TFIIIA were randomly substituted by the amino acids shown. Transcriptional activity was determined by complementation of depleted oocyte nuclear extract with 120 nM purified fusion protein as in Fig. 2. Transcription relative to the wild-type protein was determined by densitometric scanning of autoradiograms. Amino acid sequences are shown in single-letter code.

mutant proteins with changes in this block, the active mutant was the only protein that conserved a hydrophobic amino acid at position 301 (Y301V).

**Internal deletions in the conserved basic region eliminate transcription.** To determine the amino-terminal limit of the transcription-activating domain of TFIIIA, we made internal deletions to the protein immediately carboxyl to the last zinc finger. Our procedure for the construction of these mutants in the cDNA required replacing codons for the wild-type aspartic acid and proline immediately following the last zinc finger with those for alanine and glycine (see Materials and Methods for details). The protein expressed from this cDNA is called msTFIIIA (MBP fusion with a double amino acid substitution). These amino acid changes do not influence the activity of the protein in our assays. msTFIIIA showed the same DNA-binding and transcriptional activities as mTFIIIA does (Fig. 4).

Deletion of the eight amino acids (msTFIIIA  $\Delta$ 279-286) that comprise a conserved basic region in all frog TFIIAs (see Fig. 7A) eliminated transcriptional activity but had no effect on DNA binding (Fig. 1C) or RNA binding (data not shown and reference 11). Further deletions of an additional five or nine amino acids toward the C terminus (msTFIIIA  $\Delta$ 279-291 and msTFIIIA  $\Delta$ 279-295) were also transcriptionally inactive but retained DNA-binding activity. The denaturing polyacrylamide gels used for analysis of transcripts can resolve single-nucleotide differences in transcript length. The length of transcript, and therefore start site, was identical between mTFIIIA and all internal deletions, suggesting that the location of the C terminus of TFIIIA is not the dominant factor for positioning RNA polymerase III. The



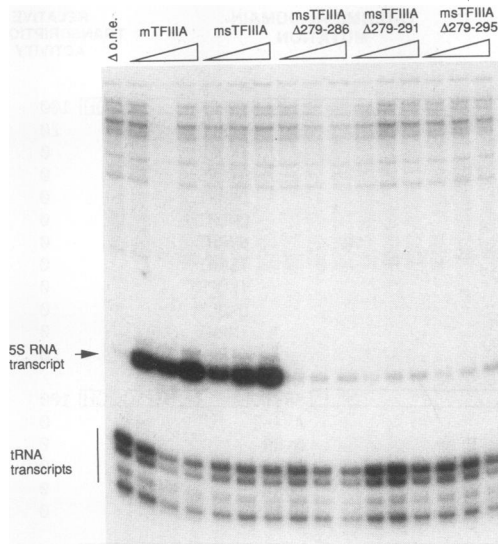


FIG. 4. Internal deletions immediately following zinc finger 9 eliminate transcriptional activity. Depleted oocyte nuclear extract was complemented with purified fusion proteins and used to transcribe a mixture of *X. borealis* somatic-type 5S RNA and *X. laevis* tRNA<sup>Met</sup> genes. Each triangle above three lanes represents increasing amounts (12, 25, and 50 nM) of the indicated purified fusion protein. Fusion protein was not added to depleted oocyte nuclear extract in the transcription reaction in lane  $\Delta$  o.n.e. Transcripts were labeled by inclusion of [ $\alpha$ -<sup>32</sup>P]CTP in the reaction and were separated by denaturing polyacrylamide gel electrophoresis.

mutant with the shortest deletion, msTFIIIA  $\Delta$ 279–286, competed for tRNA gene transcription factors as well as msTFIIIA in depleted oocyte nuclear extract. However, proteins with longer internal deletions, which include amino acids shown by random substitution to be part of the transcription-activating domain (Fig. 3), competed less well. This observation suggests the possibility of a direct interaction of the transcription-activating domain with shared transcription factors.

**The length and not the amino acid sequence of the basic region is important for transcriptional activation.** To distinguish between a direct role for the basic region in transcription or an indirect role by positioning the 15-amino-acid transcription-activating domain an appropriate distance from the DNA-binding domain, we replaced the basic-region amino acids with randomly selected amino acids. Mutagenesis was carried out by introducing biased degeneracy to nucleotides encoding the basic region. The normal nucleotide at each position in the sequence was replaced with 8% of each non-wild-type base.

A cDNA library of basic-region mutants was constructed, and 40 randomly selected clones were expressed. Of these, 25 clones yielded proteins of the predicted 82 kDa by SDS-gel electrophoresis, and 20 unique proteins were identified by DNA sequencing. The conceptual translation of their basic regions is compiled in Fig. 5A. The sequences diverge greatly from the wild-type sequence, yet all of the mutants with amino acid substitutions in this region were able to complement 5S RNA transcription in a TFIIIA-depleted oocyte nuclear extract, arguing against an important direct role for this conserved sequence. When ranked by similarity to the wild-type sequence, these substitution mutants show no correlation with transcriptional activity (Fig.

A. Mutant	Sequence		Relative Activity
	279	286	
msTFIIIA	EK RKLKE K		100
ranbr 32	-- ----D -		88
ranbr 18	-- -H--- D		50
ranbr 25	-- A-V-- -		78
ranbr 23	-- G--R- R		128
ranbr 20	V- G---- -		96
ranbr 03	-- SEV-D -		53
ranbr 04	-- -NLT- G		56
ranbr 06	-- G-V-N -		87
ranbr 16	-- WL--A -		96
ranbr 21	A- --PN- -		93
ranbr 19	-I GR--- -		83
ranbr 07	-M S-V-- A		44
ranbr 09	D- WG-I- R		47
ranbr 40	GR G---D T		51
ranbr 34	-- -Q-LH G		73
ranbr 30	-- SER-- Q		66
ranbr 39	-Y T---S N		94
ranbr 14	-E -DR-G R		83
ranbr 29	G- SN-NL N		76
ranbr 12	GT -QV-Y Y		107

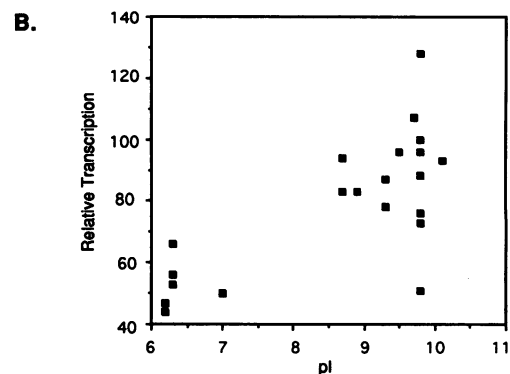


FIG. 5. The basic region of TFIIIA can be substituted with a wide range of amino acids without losing transcriptional activity. (A) Relative transcriptional activity of random substitutions in the basic region that is immediately carboxyl to zinc finger 9 (amino acids 279 to 286). Transcriptional activity by 60 nM purified fusion protein was determined by complementation of depleted oocyte nuclear extract as in Fig. 2. Transcription relative to the wild-type protein was determined by densitometric scanning of autoradiograms. Amino acid sequences are shown in single-letter code. (B) Dependence of transcriptional activity on pI of the substituted amino acids. The calculated isoelectric point of each group of eight amino acids substituted for the wild-type sequence is plotted against relative transcriptional activity as determined above.

5A). The substitutions that have highest transcriptional activity tend to be more basic, as illustrated by the plot of predicted isoelectric point for the substituted region with transcriptional activity shown in Fig. 5B. These data are consistent with the hypothesis that the amino acid sequence of the basic region is not required for transcription and that deletion of this region inhibits transcription by improperly positioning the transcription-activating domain in the initiation complex.

**The TFIIIA transcription-activating domain is position dependent.** We made insertions into the basic region to determine the effects of increasing the distance between the

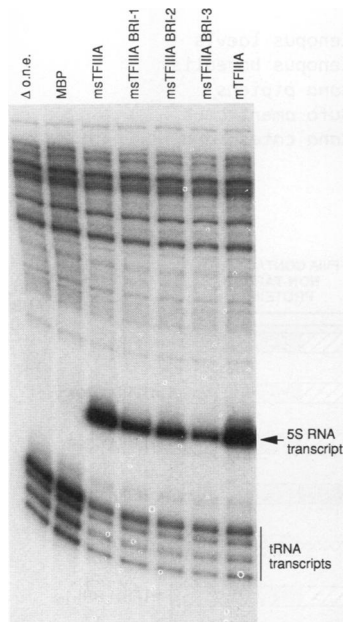


FIG. 6. The TFIIIA transcription-activating domain is sensitive to its location relative to the zinc finger domain. Depleted oocyte nuclear extract was complemented with 120 nM the indicated purified fusion proteins and used to transcribe a mixture of *X. borealis* somatic-type 5S RNA and *X. laevis* tRNA<sup>Met</sup> genes. msTFIIIA BRI-1 and BRI-2 have 10-amino-acid insertions after zinc finger 9. msTFIIIA BRI-3 has a 20-amino-acid insertion after zinc finger 9. Fusion protein was not added to depleted oocyte nuclear extract in the transcription reaction in lane  $\Delta$  o.n.e. In lane MBP, 120 nM MBP was added to depleted extract. Transcripts were labeled by inclusion of [ $\alpha$ -<sup>32</sup>P]CTP in the reaction and were separated by denaturing polyacrylamide gel electrophoresis.

transcription-activating domain and the DNA-binding domain. An oligonucleotide encoding the eight amino acids of the basic region was inserted at the unique *Ngo*MI site engineered into the msTFIIIA cDNA at the 3' end of sequences encoding zinc finger 9. Depending on the orientation of insertion of the oligonucleotide, 10 amino acids including the basic region or 10 amino acids of unrelated sequence are introduced. Three different insertion mutants were identified by DNA sequencing. BRI-1 has a single duplication of the basic region amino acids (EKLK RKEKAG); BRI-2 has an insertion of 10 unrelated amino acids (FLLQLPLLAG); BRI-3 has an insertion of 20 amino acids (FLLQLPLLAGEKLRKEKAG).

Activation of 5S RNA transcription by msTFIIIA insertion mutants is inversely proportional to the number of amino acids inserted after zinc finger 9 (Fig. 6). Insertion of 10 additional amino acids resulted in a 66% reduction in activity. Consistent with the random substitutions noted above, the nature of the amino acids appears irrelevant, as both BRI-1 and BRI-2 have the same transcriptional activity but different sequences (msTFIIIA BRI-1 and msTFIIIA BRI-2 in Fig. 6). A further reduction in transcription was observed when 20 amino acids were inserted (BRI-3). In contrast to the internal deletion mutants (Fig. 4), transcription is reduced gradually when amino acids are inserted. The transcription start site is unchanged by extension of the C terminus, again arguing against a dominant role for the TFIIIA C terminus in positioning RNA polymerase III at the start of transcription.

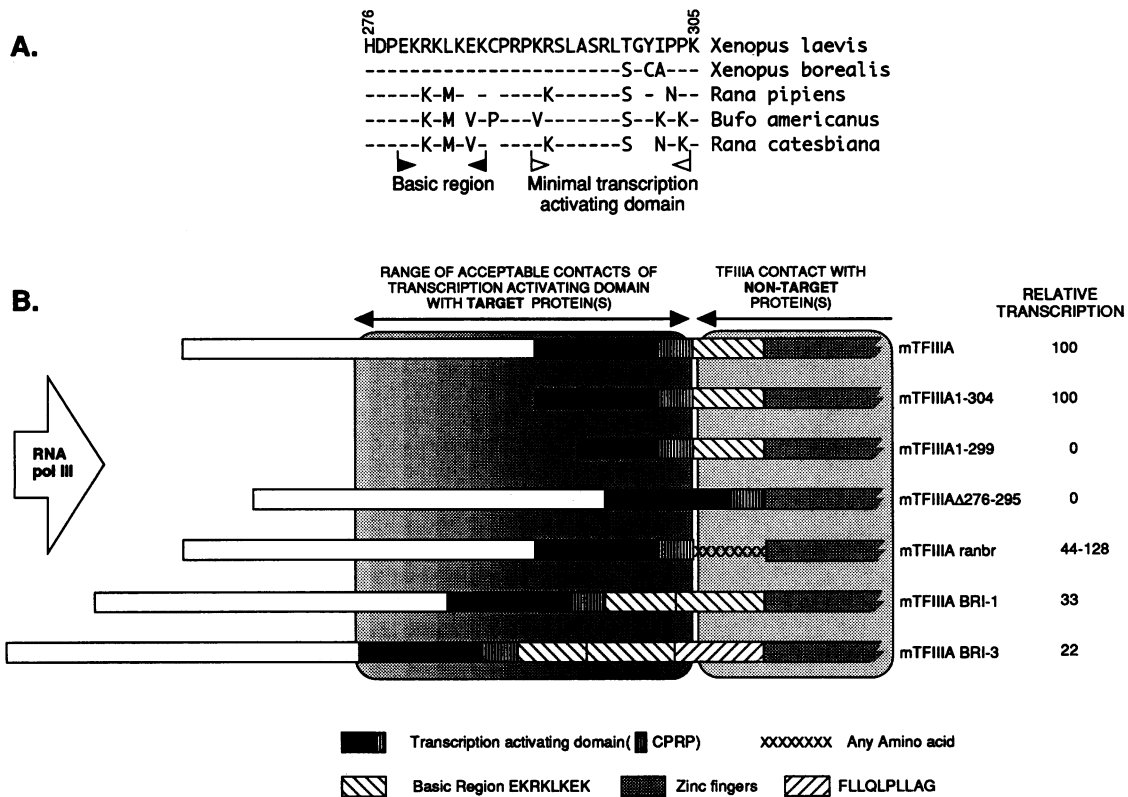
## DISCUSSION

A 14- to 18-amino-acid region in the carboxyl-terminal segment of TFIIIA is indispensable for activation of 5S RNA gene transcription. The identification of this domain derives from the results of three mutagenesis experiments. First, TFIIIA fusion proteins with defined C-terminal deletions retain full transcriptional activity when up to 40 amino acids have been deleted (mTFIIIA 1-304; Fig. 2B). Deletion of five more amino acids renders the protein inactive (mTFIIIA 1-299; Fig. 2B). Further deletions of 50, 55, and 60 amino acids are also inactive (data not shown). Second, replacement of five-amino-acid segments spanning amino acids 291 to 305 with randomly selected amino acids reveals a segment of protein that is highly sensitive to primary sequence changes (Fig. 3). Third, amino acids 277 to 286 are relatively insensitive to change, since divergent amino acid sequences can replace the wild-type sequence without dramatically decreasing transcriptional activity (Fig. 5). Therefore, we define the transcription-activating domain minimally as 14 amino acids (291 to 304), though it may comprise as many as 18 amino acids (287 to 304), since 4 amino acids, 287 to 290, were not mutated. One caveat to the use of protein expressed in *E. coli* is that incorrect folding of the polypeptide in vitro may be the cause of transcriptional inactivation. We have attempted to control for this variable by preparing a sample of mTFIIIA with each new batch of mutants. In addition, fusion proteins retain DNA-binding activity and, like the normal protein, can be stabilized by oocyte nuclear extract, indicating that their structure is not grossly perturbed. The transcription-activating region that we define, amino acids 287 to 304, is a refinement of a domain previously identified as amino acids 295 to 313. The previous definition of the activating domain was restricted by the position of convenient restriction sites for in vitro runoff transcription and translation of TFIIIA cDNA (48).

The transcription-activating domain is highly conserved among TFIIIA molecules from different frog species (Fig. 7A). Amino acid sequences on the carboxyl side of this domain diverge dramatically. Sequence conservation probably reflects functional conservation, since TFIIIA isolated from *Rana catesbiana* is able to stimulate transcription in an *X. laevis* egg extract (17). This region of TFIIIA has no apparent sequence homology with other transcription factors in the GenBank data base (release 75). The region may form a structurally isolated domain since it is flanked on both amino and carboxyl sides by proline residues, which are able to form sharp turns or flexible hinges in protein structures. Flexibility of the region could be a feature of its mechanism of action. The internal substitution mutants that we assayed cannot directly address a requirement for proline in the domain. Three basic residues (positions 289, 292, and 297) and three serines or threonines (positions 293, 296, and 299) are conserved and could be important for protein-protein interaction. If the region assumed an  $\alpha$ -helical conformation, a helical wheel projection predicts weak amphipathicity. Although hydrophobic and nonpolar amino acids (L, A, L, Y, and I) lie on one side of a putative helix, charged amino acids are positioned all around the helix. One substitution mutation that changed just two amino acids in the full-length protein (serine 296 to alanine and glycine 300 to glutamic acid) resulted in complete loss of transcription, illustrating the sensitivity of this domain to amino acid substitution and supporting an important role for the conserved serine.

The conserved basic-region amino acid sequence adjacent to the transcription domain is not required for transcription





**FIG. 7.** (A) Sequence comparison of TFIIIA proteins. Amino acid sequences are shown in single-letter code. The amino-terminal histidine of the segment shown is the C-terminal residue of zinc finger 9. References: *X. laevis* (21), *X. borealis* (26), *Rana pipiens* and *Bufo americanus* (19), and *R. catesbiana* (18). (B) Summary and model for the transcriptional properties of TFIIIA C-terminal mutants. Boundaries of productive interactions of TFIIIA with target and nontarget proteins in the transcription complex are shown (round-cornered boxes). An alignment of our TFIIIA mutants, with C termini on the left, is superimposed. Functional domains of TFIIIA and their positions in the carboxyl termini are schematically illustrated by shaded boxes. The length of each box is proportional to the number of amino acids that it represents. The gradient of shading in the target protein domain represents a range of acceptable positions for the transcription-activating region of TFIIIA; the darker shading corresponds to more transcriptionally active positions. The direction of transcription by RNA polymerase III is indicated by the open arrow. We propose that an active TFIIIA protein requires an intact transcription-activating domain that is wholly positioned within the gradient-shaded box.

activation. A surprisingly diverse range of amino acid substitutions could substitute this domain without significantly reducing transcription. One mutant (TFIIIA ranbr12) replaced six of eight amino acids, including amino acids with very different chemical properties, yet retained wild-type levels of transcription. One of the simplest explanations for these observations is that deletion of the basic region, which results in an inactive protein, places the transcription-activating domain too close to the DNA-binding domain. The threefold variation in transcription levels when randomly selected amino acids are substituted may reflect the range of different positions that the transcription-activating domain consequently occupies. The basic region is predicted to have an  $\alpha$ -helical structure. However, this structure is not a prerequisite for transcription, since calculated  $\alpha$ -helical propensity of each basic region substitution does not correlate with transcription. The chemical properties of amino acids in this region may be important, since the more active mutants tend to be more basic (Fig. 5). While this indicates a minor role for basic residues in transcription activation, the exceptions illustrate the unimportance of primary sequence.

Conservation of the primary sequence of the basic region in frog TFIIAs suggests a conserved function unrelated to transcription or DNA binding. This sequence is also con-

served in the related *Xenopus* protein p43, which binds to 5S RNA but not to the 5S RNA gene (26). However, deletion of this region does not eliminate RNA binding, and all of the basic-domain mutants bind to 5S RNA (reference 11 and data not shown). TFIIIA must enter the nucleus to activate 5S RNA transcription. It subsequently exits from the nucleus as a complex with 5S RNA (7S ribonucleoprotein) which is retained in the cytoplasm prior to ribosome biogenesis (23, 39). The basic region may fulfill a requirement for a nuclear localization signal. Nuclear localization signals are varied but tend to have at least one element with three or four contiguous basic residues (14).

The mechanism of activation of 5S RNA transcription by TFIIIA is unknown but is probably mediated through protein-protein interaction. All of the TFIIIA proteins with mutations in the transcription-activating domain bind to 5S DNA, so it is unlikely that the activating domain makes direct contact with DNA. Furthermore, DNA contacts by each zinc finger on the ICR are known and account for the entire DNase I-protected region (24). However, direct contact of the C terminus with DNA induced through other components of the transcription complex cannot be excluded. The transcription-activating domain could function either by recruiting or positioning a transcription factor or by

acting as a trigger. As a trigger, the domain may induce a conformational change in its target that elicits a new function. If the transcription-activating domain functions as a conformational trigger, transcriptionally silent TFIIIA mutants should still recruit TFIIC and TFIIB.

The ability of transcriptionally inactive TFIIIA mutants to bind factors shared with tRNA genes suggests that components of TFIIB or TFIIC are recruited. However, some C-terminal deletion mutants (e.g., mTFIIIA  $\Delta$ 1-299 and  $\Delta$ 279-286) are better able to compete with tRNA genes for transcription factors than are others (e.g., msTFIIIA  $\Delta$ 279-295). This difference suggests that there is more than one way to inactivate transcriptional activation through the C terminus. The stabilizing activity in oocyte nuclear extract has been shown to be TFIIC (50). DNA binding by transcriptionally inactive mutants is stabilized in oocyte nuclear extract. Protein-protein interactions with the zinc fingers could account for this stabilization. A role for zinc fingers in transcriptional activation distinct from DNA binding has been suggested (13). Complex stability is not a prerequisite for transcription (51), so differences in stability of complexes formed with C-terminal mutants are not necessarily reflected by changes in transcriptional activation.

TFIIB is the crucial factor for RNA polymerase recognition of the 5S RNA promoter in *S. cerevisiae*. The order of recruitment of factors to the 5S RNA gene promoter (TFIIA, TFIIC, and then TFIIB) argues against direct recruitment of TFIIB by TFIIA. However, TFIIA could dictate the start site of transcription by positioning TFIIB following TFIIB's recruitment to the complex by TFIIC. Deletion studies of the 5S RNA gene 5' to the ICR have shown that RNA polymerase III selects a start site that is approximately 50 bp upstream of the TFIIA-binding site regardless of the intervening DNA sequence (42). If the transcription-activating domain plays a dominant role in positioning RNA polymerase III, either directly or through TFIIB, then TFIIA mutants that change the amino acid distance from this domain to the DNA-binding domain could be expected to cause a change in the position of the transcription start site and therefore transcript length. However, transcript length did not change by a single nucleotide when 20 amino acids were inserted between the transcription-activating domain and the DNA-binding domain.

The function of the TFIIA-activating domain appears to be distinct from activators of RNA polymerase II in that it is very sensitive to its position relative to the DNA-binding domain. Insertion of amino acids that place the transcription-activating domain further from the DNA-binding domain has less dramatic consequences than deletion of amino acids in the same region. Whereas removal of 8 amino acids eliminated transcription, insertion of 20 amino acids reduced transcription only fivefold.

A model for the function of the transcription-activating domain derived from our observations is presented in Fig. 7B. The interaction of TFIIA with two classes of proteins, those that are the target for the transcription-activating domain and nontarget proteins, i.e., all other proteins in the transcription complex, is considered. Our data suggest that the entire activating domain of 14 amino acids is required to contact its target. We propose that deletion or insertion of amino acids decreases transcription because the transcription-activating domain falls out of range of productive interaction with its target, represented by the gradient-shaded box in Fig. 7. One physical mechanism that could account for the rapid loss of transcription by deletions to its amino-terminal side would be steric masking of target binding by a

nontarget protein such that the transcription-activating domain cannot find its target (mTFIIA ranbr in Fig. 7). An example of a nontarget protein could be a component of the transcription complex that interacts with the C-terminal zinc fingers of TFIIA. Masking the activation domain as a consequence of internal deletions is most probable if nontarget protein binds prior to the target. Similarly, increasing the number of amino acids between zinc fingers and the transcription-activating domain places the domain out of the range of interaction with its target (mTFIIA BRI-1 and BRI-3 in Fig. 7). However, in this case, the consequence is a gradual loss of transcription, since the transcription-activating domain is not masked by nontarget proteins.

Distinguishing between the functions of the transcription-activating domain as a trigger or in transcription complex assembly will require identification of the transcription factors recruited into an initiation complex by our mutants. In the course of these studies, we have constructed four cDNA libraries of random mutations within the C terminus of TFIIA. Development of an efficient screen for active mutants will permit a better definition of the critical amino acids in the transcription-activating domain and a better understanding of its function.

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