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Species variation in transcription factor IIIA

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ABSTRACT

Species variation in transcription factor IIIA (TFIIIA) was examined by comparing the abilities of TFIIIAs isolated from different Xenopus and Rana species to 1) bind rabbit anti-Xenopus laevis TFIIIA IgG, 2) specifically interact with the Xenopus borealis somatic 5S RNA gene, and 3) promote transcription of the Xenopus borealis 5S RNA gene in vitro. In immunoblot assays, Rana catesbeiana or Rana pipiens TFIIIA did not react readily with rabbit anti-Xenopus laevis TFIIIA IgG (assayed with anti-rabbit $F(ab')_2$ fragment conjugated with alkaline phosphatase) whereas Xenopus borealis TFIIIA exhibited similar reactivity with this IgG as Xenopus laevis TFIIIA. When compared to Xenopus TFIIIAs, Rana TFIIIAs exhibited similar interactions with the 3' portion of the intragenic control region of the Xenopus 5S RNA gene (to residue +78 on the coding strand and up to and including +74 on the non-coding strand, nucleotides protected from DNase I digestion by the N-terminal half of Xenopus TFIIIA) and incomplete interactions with the remaining 5' portion of the control region (nucleotides protected from DNase I digestion by the C-terminal half of Xenopus TFIIIA). In a Xenopus laevis unfertilized egg extract, Rana catesbeiana and Rana pipiens TFIIIAs promoted transcription of the Xenopus borealis somatic 5S RNA gene less efficiently than Xenopus laevis and Xenopus borealis TFIIIAs.

INTRODUCTION

The initiation of transcription of the 5S ribosomal RNA genes during oogenesis in Xenopus laevis requires RNA polymerase III and transcription factors TFIIIA, TFIIIB, and TFIIIC (1,2). TFIIIA binds to the intragenic control region of the 5S RNA gene, protecting from DNase I digestion residues +43 to +96 on the coding strand and residues +45 to +95 on the non-coding strand (1,3,4). TFIIIA induces DNase I hypersensitivity at positions +93, +74, and +62 on the non-coding strand and +43 on the coding strand (1). The N-terminal half of Xenopus laevis TFIIIA interacts with the 3' portion of the 5S RNA gene ICR and the C-terminal half of the protein interacts with the 5' portion of the ICR (5). In immature oocytes, TFIIIA is found associated with oocyte 5S RNA in a 7S particle (6,7,8).

TFIIIA requires zinc for specific binding to the 5S RNA gene and transcription promotion in vitro (9). Analysis of the amino acid sequence of

TFIIIA (10) revealed the presence of 9-11 repetitive elements of about 30 amino acids each, with a cys...cys... his...his motif believed to be involved in coordination of a zinc atom (11,12). The TFIIIA gene from Xenopus laevis encodes nine potential zinc binding sites (termed "zinc fingers") comprising the N-terminal 80% of the protein; the N-terminal fingers correspond to the first six exons in the gene (13). The structural integrity of the N-terminal zinc fingers of TFIIIA is required for the initiation of specific interactions with the 3' region of the 5S RNA gene ICR (5,14). Amino acid repeats, similar in sequence to the TFIIIA zinc finger, have been found in the amino acid sequences of a variety of gene regulatory proteins (15). Zinc has also been shown to be required for the function of the yeast Gal4 transcription factor, HeLa cell SpI, and glucocorticoid receptor (16,17,18). Zinc binding sites appear to be an evolutionary thread connecting many eukaryotic gene regulatory proteins.

The amino acid sequence for TFIIIA is significantly polymorphic within the Xenopus laevis species (13). It was reasoned therefore that even greater divergence in structure and function might occur between TFIIIA proteins from different amphibian species. To begin to understand species variation in TFIIIA, we have isolated TFIIIAs from different species within frog genera Xenopus and Rana and compared their immunological, DNA binding, and transcription promotion properties. Results from these comparative analyses indicate that TFIIIA is less conserved between Xenopus and Rana genera than within these genera and, moreover, suggest greater divergence exists between the C-terminal halves of Rana and Xenopus TFIIIA than the N-terminal halves.

MATERIALS AND METHODS

Purification of TFIIIA-containing 7S Particles

Ovaries, excised from approximately twenty immature Xenopus laevis, X. borealis, Rana catesbeiana (American bullfrog), or R. pipiens (leopard frog), were homogenized in 6 ml of 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM phenylmethyl-sulfonyl fluoride at 0 °C. 7S particles, composed of TFIIIA and oocyte 5S RNA, were isolated by glycerol gradient centrifugation of the respective immature ovarian homogenates followed by DEAE-cellulose chromatography of the 7S fraction (19). The Xenopus species were obtained from Nasco (Wisconsin) and the Rana species were obtain from the Wm. Lemberger Co. (Wisconsin). TFIIIA was released from 5S RNA by digesting 7S particles (20 μg/ml) with RNase A (20 μg/ml) for 30 min at 23 °C in 100 μl of 20 mM Tris-HCl, pH 7.5, 320 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM dithiothreitol (20). TFIIIA samples prepared this way were stored on ice and used immediate-

ly. Protein concentrations were determined by the method of Bradford (21) using bovine serum albumin as a standard.

Non-denaturing and SDS Polyacrylamide Gel Electrophoresis

7S particles, isolated from X. laevis, X. borealis, R. catesbeiana, or R. pipiens immature ovaries, were electrophoresed through non-denaturing, 6% polyacrylamide gels (19:1, acrylamide: bisacrylamide, 80 mM Tris base, 80 mM boric acid, 1 mM EDTA, pre-electrophoresed 1 hr at 130 V, ref. 22) for 2.5 hr at 130 V (23 °C) in the Tris-borate-EDTA buffer. Just prior to gel loading, TFIIIA samples (2 µg protein) were suspended in 20 µl of 10 mM EDTA, 10% glycerol, and 0.01% bromophenol blue. X. laevis oocyte 5S RNA and tRNA, isolated by phenol extraction of 42S particles (8) were used as electrophoresis markers. The electrophoresed gel was stained with ethidium bromide (1 µg/ml in deionized water) for 15 min, rinsed three times in deionized water, and photographed on a 302 nm lightbox with Polaroid type 665 film. TFIIIA-containing 7S particles from Xenopus and Rana species were also subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (19). 7S particles (protein amount given in Fig. legends) were denatured by adding equal volumes (50 µl) of SDS sample buffer and heating at 95 °C for 10 min. Myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa) obtained from Sigma Chem. Co., were used as molecular weight markers. Gels were electrophoresed 2-4 hrs at 140 V in 25 mM Tris base, 0.2 M glycine, and 0.1% SDS. Gels were stained with 0.2% Coomassie blue R, 40% methanol, 7.5% acetic acid and destained in this solution minus the Coomassie blue. Destained gels were photographed on a white light box with Polaroid 665 film.

Immunoblotting of TFIIIA

TFIIIAs were subjected to SDS PAGE as described above. The gel was equilibrated in transfer buffer (80 mM Tris-glycine, pH 8.3, 20% methanol) and the proteins were transferred onto nitrocellulose (23). The nitrocellulose blot was soaked in phosphate-buffered saline (PBS) plus 0.5% nonfat dry milk followed by washing with 1X PBS and 0.05% Tween 20. The blot was then incubated with rabbit antiserum against TFIIIA (1:500 dilution) in 1% bovine serum albumin. TFIIIA immunization of New Zealand white rabbits was performed by injecting 50 µg of Xenopus laevis TFIIIA into each popiteal lymph node of a white New Zealand rabbit followed by 3 bi-weekly intramuscular injections (hind legs) of 25 µg of antigen (P. Haab, Pocono Laboratories, Canadensis, PA). Serum was prepared from bleedings beginning at 12 weeks post primary immunization. The immunoblot was adsorbed with alkaline phosphatase-conjugated, sheep

anti-rabbit IgG F(ab')$_2$ fragment followed by incubation in β -naphthyl acid phosphate stain (24). Protein bound to nitrocellulose was detected by staining with 0.1% naphthol blue black in 25% isopropanol and 10% acetic acid followed by destaining in isopropanol/acetic acid.

End-labeling of the Xenopus 5S RNA Gene and DNase I Protection Studies

Plasmid pXbs201, which contains the Xenopus borealis somatic 5S RNA gene (4), was linearized and end-labeled by EcoRI digestion followed by filling in the 5' overhangs with reverse transcriptase and [α^{32}P] dATP (22). The linearized plasmid was then cut with BamHI and the resultant 303 bp fragment, containing the 120 bp 5S RNA gene end-labeled on the coding strand, was isolated by agarose gel electrophoresis and electroelution. This insert was labeled on its non-coding strand by first cutting pXbs201 with BamHI, filling in the overhang with dGTP and [α^{32}P]dATP, and recutting with EcoRI. The specific activities of the end-labeled fragments were determined by quantitating DNA from A_{260} and [^{32}P] by scintillation counting. Interactions of TFIIIA isolated from X. laevis, X. borealis, R. catesbeiana, and R. pipiens with the end-labeled, Xenopus 5S RNA gene were monitored by DNase I protection (25). TFIIIA and the end-labeled 5S RNA gene-containing fragment, at concentrations indicated in the Fig. legends, were incubated 10 min at 23 °C in 20 µl of 20 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 7 mM $MgCl_2$, 0.5 mM DTT, and 0.01% Nonidet P-40. DNase I was added to a final concentration of 2 µg/ml for 1 min followed by addition of 100 µl of 30 mM sodium citrate (pH 7.0 with HCl), 300 mM NaCl, 0.5% SDS, and 30 µg/ml sonicated salmon sperm DNA. DNA was ethanol precipitated, dried, resuspended in 5 µl formamide sample buffer (7), heated 2 min at 95 °C, and electrophoresed through 7% polyacrylamide gel (7 M urea, 20:1, acrylamide:bisacrylamide in Tris-borate-EDTA buffer, prerun 4 hr at 400V) until the xylene cyanol marker had migrated three quarters down the gel (2hr at 2000V). Gel autoradiography was performed with Kodak X-OMAT AR film and a Dupont Cronex intensifying screen and exposed overnight at -70 °C.

In Vitro Transcription of the Xenopus 5S RNA gene

TFIIIA-dependent transcription of the Xenopus borealis 5S RNA gene was performed in an unfertilized egg extract (26,27). Oocyte-positive Xenopus laevis (Nasco, Ft. Atkinson, WI) were injected in the dorsal lymph sacs with 1000 units of human chorionic gonadotropin and the shed eggs were collected overnight in modified Barth's solution (15 mM HEPES, pH 7.6, 88 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MgSO_4$, 0.5 mM Na_2HPO_4, and 2 mM $NaHCO_3$). Eggs were dejellied by gently swirling in 2% cysteine-HCl, pH 7.8, and rinsed 4 times in modified Barth's solution and 2 times in distilled water. The egg

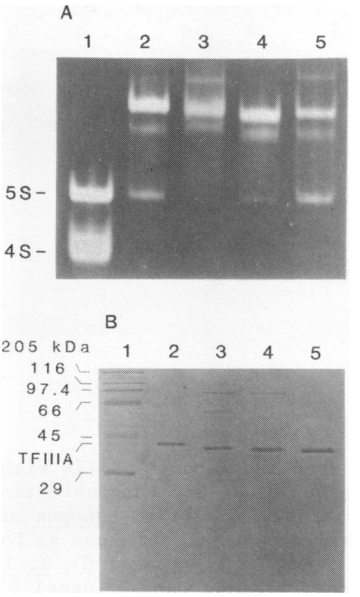

Fig. 1. Isolation of TFIIIA from other frog species. TFIIIA isolation, non-denaturing gel electrophoresis, and SDS PAGE were performed as described in MATERIALS AND METHODS. A. Non-denaturing PAGE of 2 μg (protein) of X. laevis, X. borealis, R. catesbeiana, and R. pipiens 7S particle (lanes 2-5); lane 1 contains 5S and 4S RNA markers. B. SDS PAGE of 4 μg (protein) of the 7S particle in lanes 2-5 as in A; lane 1 contains the molecular weight markers described in MATERIALS AND METHODS.

mass, containing only interstitial water, was homogenized by a single stroke in a precooled teflon-glass homogenizer and the egg extract was prepared as described previously (27). Transcription reactions (50 μl) contained 10 mM HEPES, pH. 7.5, 70 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM ATP, GTP, CTP, 0.02 mM UTP, 2 μCi [α ^{32}P] UTP, 2 ug pXbS201, and 1 or 4 μg of TFIIIA-containing 7S particles. Reactions were incubated for 2 hrs at 23 °C, stopped by addition of 3 μl 10% SDS, and then incubated with 1 μg of proteinase K for an additional 30 min. Following phenol extraction and ethanol precipitation, the RNA samples were resuspended in 50% deionized formamide, heated 30 sec at 90 °C, and electrophoresed 2.5 hrs at 170 volts through a 10% acrylamide gel (19:1 acrylamide:bis-acrylamide) in 80 mM Tris, 80 mM boric acid, 1 mM EDTA buffer. Gel autoradiography was performed as described above.

RESULTS

Isolation of 7S particles From Other Frog Species

TFIIIA constitutes as much as 10% of the cellular protein in Xenopus

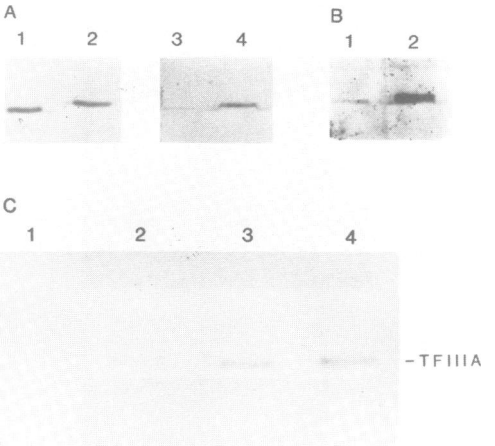

Fig. 2. Immunoblotting of Rana and Xenopus TFIIIA with anti-X. laevis TFIIIA antisera. TFIIIA isolation, SDS PAGE, and immunoblotting were performed as described in MATERIALS AND METHODS. 4 μg of Xenopus or Rana TFIIIA were electrophoresed and transferred to nitrocellulose as follows: lanes 2 and 4 (panel A), lane 2 (panel B), and lane 4 (panel C), X. laevis TFIIIA; lanes 1 and 3 (panel A), lane 1 (panel B), and lane 2 (panel C), R. catesbeiana TFIIIA; lane 3 (panel C), X. borealis TFIIIA; lane 1 (panel C), R. pipiens TFIIIA. Lanes 1 and 2 in panel B were immunoblotted with a different anti-X. laevis TFIIIA antiserum than used in lanes 3 and 4, panel A, and panel C. The nitrocellulose filter in lanes 1 and 2, panel A, was stained only with 0.1% naphthol blue black.

laevis immature oocytes (28) and is purified as a 7S particle complexed with oocyte 5S RNA by glycerol gradient centrifugation followed by DEAE-cellulose chromatography (19). This procedure was used to isolate putative 7S particles from the immature ovarian tissue of X. borealis, R. catesbeiana (American bullfrog), and R. pipiens (leopard frog). Glycerol gradient centrifugation of immature ovarian homogenates from these three frog species revealed a peak of A_{260} absorbing material in similar fractions to where the X. laevis 7S particle is observed (gradient not shown). Various 7S fractions were electrophoresed through a non-denaturing polyacrylamide gel (stained with ethidium bromide, Fig. 1A). Lane 2 contains the X. laevis 7S particle and lanes 3, 4, and 5 contain the 0.32 M KCl DEAE fractions from X. borealis, R. catesbeiana, and R. pipiens. All the DEAE fractions contain a major ethidium fluorescing band in a similar migration position (7S) to that of the X. laevis 7S particle. The weakly fluorescing band above/below the major 7S species most likely is due to aggregation/degradation. A small amount of free 5S RNA is also observed in these 7S particle preparations (fluorescing band co-migrating with 5 S RNA marker). Fig. 1B is a photograph of SDS PAGE of 7S particles from X. laevis,

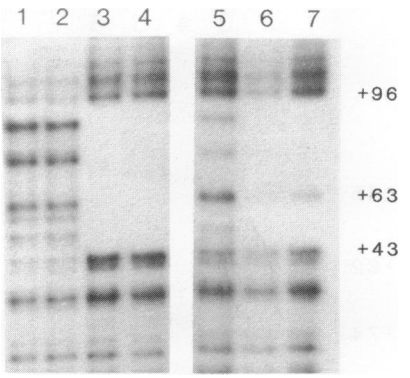

Fig. 3. Interaction of Xenopus borealis TFIIIA with the Xenopus 5S RNA gene end-labeled on the coding strand. TFIIIA isolation, DNA end-labeling, and DNase I protection were performed as described in MATERIALS AND METHODS. The DNase I protection reaction contained $1x10^{-9}$M of the end-labeled 5S RNA gene and 2.5, 5, and $10x10^{-9}$M TFIIIA (lanes 2-4, X. laevis and lanes 5-7, X. borealis). Lane 1 exhibits the DNase I digestion pattern of the end-labeled 5S RNA gene in the absence of TFIIIA. Nucleotide positions on the coding strand of the Xenopus 5S RNA gene are relative to the +1 transcription initiation site and were determined previously (1).

X. borealis, R. catesbeiana, and R. pipiens (lanes 2-5) stained with coomassie blue; lane 1 exhibits molecular weight markers. The molecular weight of X. laevis TFIIIA is about 40 kDa (major Coomassie stained band, lane 2). The molecular weights (electrophoretic mobilities) of the other frog TFIIIAs (Xenopus borealis, Rana catesbeiana, Rana pipiens, major bands, lanes 3-5) are very similar to that of Xenopus laevis TFIIIA although a slight decrease in molecular weight (increased mobility) is observed.

Immunoblot Analyses of Xenopus and Rana TFIIIA

Immunoblot methodology is capable of detecting structural similarities or differences between the same protein from different species (23). To determine whether the structure of TFIIIA is strictly conserved between or within frog genera (i.e., bind an anti-TFIIIA antibody equally well), this protein from different species of the Xenopus and Rana genera was subjected to denaturing SDS PAGE, transferred to nitrocellulose, and immunoblotted with a rabbit antiserum against X. laevis TFIIIA (Fig. 2). Lanes 3 and 4 in Fig. 2A contain TFIIIA from R. catesbeiana and X. laevis immunoblotted with rabbit antiserum against X. laevis TFIIIA (diluted 1 to 500). X. laevis TFIIIA reacted with significantly more anti-TFIIIA antibody than R. catesbeiana TFIIIA as evidenced by the greater density of alkaline phosphatase-dependent stain in lane 4. The light band in lane 3 is a brownish color in the orginal

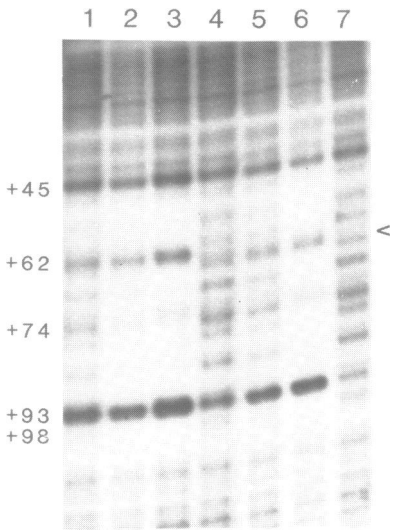

Fig. 4. Binding of Xenopus borealis TFIIIA to the Xenopus 5S RNA gene end-labeled on the non-coding strand. DNase I protection reactions are identical to that described in the Fig. 3 legend except that the Xenopus 5 S RNA gene was end-labeled on the non-coding strand. X. laevis TFIIIA, lanes 1-3; X. borealis TFIIIA, lanes 4-6. Lane 7 exhibits the DNase I digestion pattern of the end-labeled, non-coding strand of the 5S RNA gene in the absence of TFIIIA. Nucleotide positions on the non-coding strand of the Xenopus 5S RNA gene are relative to +1 transcription initiation site. The arrow in the right hand margin marks the shift in the +62 DNase I digestion site.

blot rather than the reddish color characteristic of this phosphatase-dependent stain (lane 4); the brownish color is observed with non-specific proteins in this assay. Lanes 1 and 2 contained identically electrophoresed samples as exhibited in lanes 3 and 4 but stained with naphthol blue black to demonstrate the presence of equal amounts of protein bound to the nitrocellulose filter. Fig. 2B contains R. catesbeiana and X. laevis TFIIIA immunoblotted with another rabbit anti-X. laevis TFIIIA antiserum prepared in similar fashion to that used in panel A (kindly provided by P. Thomas, Oklahoma Medical Research Foundation). As observed in panel A, the X. laevis TFIIIA (lane 2) is reacting with an anti-TFIIIA antiserum (diluted 1 to 500) to a significantly greater extent than the R. catesbeiana protein (lane 1); in the original blot, the light band in lane 1 panel B is more brownish in color rather than the characteristic red (lane 2).

To assay for conservation of TFIIIA structure within frog genera, TFIIIA from two species of the Xenopus genus (X. laevis, X. borealis) and two

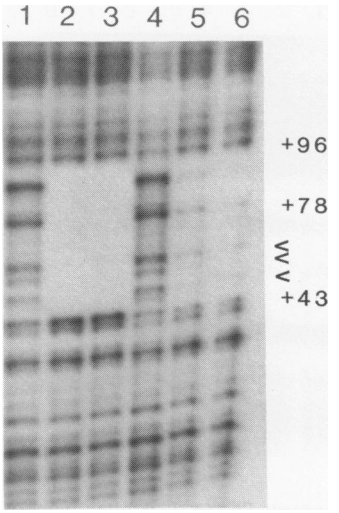

Fig. 5. Rana TFIIIA-dependent DNase I protection of the Xenopus 5S RNA gene end-labeled on the coding strand. TFIIIA isolation and DNase I protection were performed as described in the Fig. 3 legend. Lanes 1-3, 2.5, 5.0, and $10x10^{-9}$ M X. laevis TFIIIA; lanes 4-6, 5.0, 10, and $20x10^{-9}$ M R. catesbeiana TFIIIA. Arrows in the right hand margin (between residues +43 and +78) mark additional sites of reduced DNase I protection.

from the Rana genus (R. catesbeiana, and R. pipiens) were immunoblotted with anti-X. laevis antiserum (Fig. 2C). TFIIIA from two species of the Xenopus genus (lanes 3 and 4, X. borealis and X. laevis) bind anti-X. laevis TFIIIA antibody equally well as evidenced by the similar density of alkaline phosphatase-dependent stain over the two proteins. In contrast, TFIIIA from two species of the Rana genus (lanes 1 and 2, R. pipiens and R. catesbeiana) fails to interact significantly with this antiserum as evidenced by the lack of alkaline phosphatase-dependent stain over these proteins. The light band present in each of these two lanes is more brown in the original blot rather than the pink color characteristic of the phosphatase-dependent stain. These immunoblot results indicate the structure of TFIIIA is more conserved within frog genera than between frog genera.

Interaction of Xenopus and Rana TFIIIA with the Coding and Non-coding Strands of the Duplex Xenopus 5S RNA Gene

Xenopus laevis TFIIIA projects itself from N to C terminus in the 3' to 5' direction on the intragenic control region of the Xenopus 5S RNA gene (5,14). Species variation in protein-DNA interactions observed by DNase I digestion along the Xenopus control region might be localized to a region of

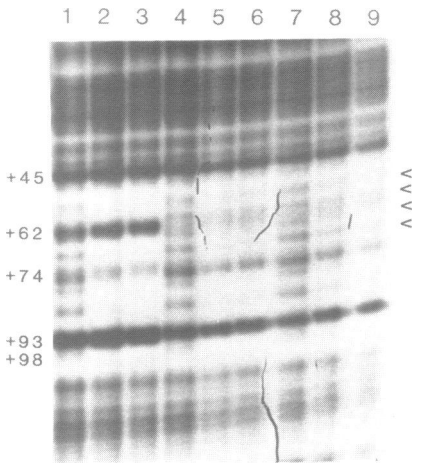

Fig. 6. Rana TFIIIA-dependent DNase I protection of the Xenopus 5S RNA gene end-labeled on the non-coding strand. Lanes 1-3, 2.5, 5.0, and 10×10^{-9} M X.laevis TFIIIA; lanes 4-6, 5.0, 10, and 20×10^{-9} M R. catesbeiana TFIIIA; lanes 7-9, 5.0, 10, and 20×10^{-9} M R. pipiens TFIIIA. Arrows in the right hand margin (between residues +45 and +74) mark additional sites of reduced DNase I protection.

the TFIIIA molecule. Fig. 3 is an autoradiogram of a DNase I protection experiment in which increasing concentrations of X. laevis (lanes 2-4) or X. borealis (lanes 5-7) have been added to the Xenopus 5S RNA gene end-labeled on its coding strand, followed by DNase I addition. Lane 1 illustrates the DNase I digestion pattern of the end-labeled gene in the absence of TFIIIA. In lanes 3 and 4, complete protection of the 5S RNA gene from nucleotides +43 to +96 on the coding strand is afforded by X. laevis TFIIIA. Complete protection is also observed in lanes 6 and 7 by the X. borealis TFIIIA except at nucleotide +63. Fig. 4 illustrates the DNase I digestion pattern on the non-coding strand of the Xenopus 5S RNA gene in the presence of increasing concentrations of X. laevis (lanes 1-3) or X. borealis (lanes 4-6) TFIIIA. The patterns (lanes 3 and 6) are nearly identical with both TFIIIAs affording protection from nucleotides +98 to +45 and inducing DNase I hypersensitivities at positions +93, +74, and around +62. Upon close inspection, the hypersensitive site at +62 (marked in right margin) is shifted down probably one nucleotide to +63 in the presence of X. borealis TFIIIA; this is the same base-pair altered by X. borealis TFIIIA on the coding strand (Fig. 3, lane 7). Except for this one anomaly, all other interactions of X. borealis and X. laevis TFIIIA with the Xenopus 5S RNA gene (as judged by DNase I protection) appear identical.

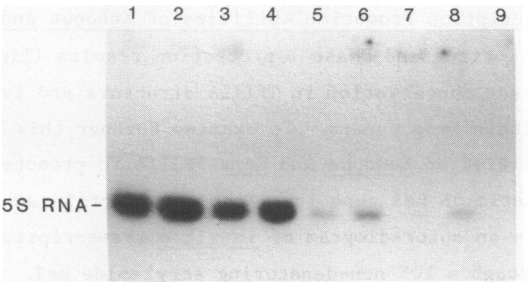

Fig. 7. Xenopus and Rana TFIIIA-dependent transcription of the Xenopus borealis somatic 5S RNA gene. TFIIIA isolation, in vitro transcription, gel electrophoresis, and autoradiography were performed as described in MATERIALS AND METHODS. Transcription reactions electrophoresed in lanes 1 and 2 contained 1 and 4 μg of X. laevis TFIIIA, lanes 3 and 4, 1 and 4 μg of X. borealis TFIIIA, lanes 5 and 6, 1 and 4 μg of R. catesbeiana TFIIIA, lanes 7 and 8, 1 and 4 μg of R. pipiens TFIIIA, lane 9, no TFIIIA.

Fig. 5 is an autoradiogram of a DNase I protection experiment comparing the ability of Xenopus TFIIIA (lanes 1-3) and R. catesbeiana TFIIIA (lanes 4-6) to protect the coding strand of the Xenopus 5S RNA gene from DNase I digestion. TFIIIA isolated from the American bullfrog fully protects the coding strand of the Xenopus 5S RNA gene from DNase I digestion from nucleotides +96 up to +78 (Fig. 5, lane 6). As evidenced by the diminished intensities of the end-labeled fragments, partial DNase I protection by R. catesbeiana TFIIIA is observed from nucleotides +78 to +43; very little DNase I hypersensitivity is observed at +43. Fig. 6 illustrates the DNase I digestion patterns of the end-labeled non-coding strand in the Xenopus 5 S RNA gene in the presence of increasing concentrations of X. laevis (lanes 1-3), R. catesbeiana (lanes 4-6), and R. pipiens (lanes 7-9) TFIIIA. Protection of the Xenopus 5 S RNA gene by the Rana TFIIIAs appears normal from nucleotides +98 up to +74, including the induction of DNase I hypersensitivity at nucleotides +93 and +74. The Rana proteins only partially protect nucleotides +74 to +45 and do not induce the prominent DNase I hypersensitivity at nucleotide +62 as does X. laevis TFIIIA (compare lanes 2 and 3 with lanes 5 and 6 and lanes 8 and 9). These diminished protein-DNA interactions exhibited by the Rana TFIIIAs on the coding and non-coding strands of the duplex Xenopus 5S RNA gene (as deduced by DNase I digestion) from nucleotides +74 to +45 are indicative of structural variability between the C-terminal halves of the Xenopus and Rana TFIIIAs. The overall affinities of the Xenopus and Rana TFIIIAs for the 3' portion of the Xenopus 5S RNA gene ICR appear similar in that DNase I protection of this region is attained at similar protein concentrations.

Comparison of Transcription Promotion Abilities of Xenopus and Rana TFIIIA

The immunoblotting and DNase I protection results (Figs. 2-6) are consistent with lesser conservation in TFIIIA structure and function between frog genera than within frog genera. To examine further this species variation in TFIIIA, the abilities of Xenopus and Rana TFIIIA to promote transcription of the X. borealis somatic 5S RNA gene in a Xenopus unfertilized egg extract was analyzed. Fig. 7 is an autoradiogram of in vitro transcription reactions electrophoresed through a 10% non-denaturing acrylamide gel. If no exogenous TFIIIA is added to this extract, 5S RNA transcription is not detected from the plasmid carrying the Xenopus borealis somatic 5S RNA gene (lane 9). The Xenopus TFIIIAs (X. laevis, lanes 1 and 2, and X. borealis, lanes 3 and 4) promote transcription of the Xenoups borealis somatic 5S RNA gene about equally well as judged by the similar intensities (incorporation of [α ^{32}P] UTP) of the 5S RNA bands. The Rana TFIIIAs (R. catesbeiana, lanes 5 and 6, and R. pipiens, lanes 7 and 8) promote transcription of the Xenopus borealis somatic 5S RNA gene in this unfertilized egg extract but at a considerably reduced efficiency than the Xenopus proteins as judged by the diminished intensities of the 5 S RNA bands in these lanes.

DISCUSSION

Because of the apparent evolutionary relationship between Xenopus laevis TFIIIA and other eukaryotic gene regulatory proteins, it would be informative to identify and analyze any species variation in TFIIIA. Toward this goal, we have isolated TFIIIAs from four frog species-Xenopus laevis, Xenopus borealis, Rana catesbeiana (American bullfrog), and Rana pipiens (leopard frog)-and compared their abilities to bind anti-X. laevis TFIIIA antibody (assayed by immunoblotting, Fig. 2), bind the ICR of the X. borealis somatic 5S RNA gene (analyzed by DNase I footprinting, Figs. 3-6, and to promote transcription of the Xenopus 5S RNA gene (assayed in a Xenopus unfertilized egg extract, Fig. 7). The immunoblotting experiments revealed that the Rana TFIIIAs reacted considerably less with anti-X. laevis TFIIIA antisera than Xenopus TFIIIAs; DNase I protection analyses demonstrated the Rana TFIIIAs afforded less protection of the 5' half of the Xenopus 5S RNA gene ICR than the Xenopus TFIIIAs; In vitro transcription analysis demonstrated that Rana TFIIIAs were less efficient at promoting transcription of a Xenopus 5S RNA gene than the Xenopus TFIIIAs. These independent analyses demonstrate that significant species variation exists in TFIIIA and the variation is greater between frog genera than within frog genera.

Although more direct evidence is lacking at this time, the immunoblotting, DNase I protection, and in vitro transcription experiments comparing the Rana and Xenopus TFIIIAs point indirectly to lesser conservation in the C-terminal halves of the proteins than the N-terminal halves because: 1) anti-X. laevis TFIIIA antiserum does not readily react with the intact Rana TFIIIAs (probably denatured from SDS PAGE) or the N-terminal half of the X. laevis TFIIIA (probable denatured form, blot not shown); 2) Rana TFIIIAs adequately protect the 3' portion of the ICR of the Xenopus 5S RNA gene (region protected by the N-terminal half of Xenopus TFIIIA) from DNase I digestion but do not adequately protect the 5' portion of the ICR (region protected by the C-terminal half of Xenopus TFIIIA); 3) Rana TFIIIAs do not readily promote transcription of Xenopus 5S RNA genes, a function dependent in part on the C-terminal region of Xenopus TFIIIA (5). Less conservation in the C-terminal half of TFIIIA is also supported by the observations that 1) the intron-exon structure of the gene coding for this region of the protein lacks the regularity observed in those sequences coding for the N-terminal half and 2) polymorphism in the amino acid sequence of X. laevis TFIIIA is observed in the C-terminal 10 kDa of TFIIIA (13). Most of the strong contacts between Xenopus TFIIIA and the Xenopus 5S RNA gene are clustered between +80 and +90 on the non-coding strand whereas sequence divergence between the X. laevis and X. borealis 5S DNA is localized to the 5' portion of the ICR (29). Likewise, the sequences of Rana oocyte 5S RNAs in the +80 to +90 region (and by deduction the corresponding nucleotide positions on the Rana 5S DNAs) are identical to Xenopus 5S RNAs whereas the 5' regions are more divergent (Gaskins and Hanas, manuscript in preparation). Proper contact between the N-terminal half of TFIIIA and the 3' portion of the intragenic control region may be of such necessity that greater conservation of the N-terminal half of the protein and the 3' region of the 5S DNA ICR has occurred.

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