Volume 281, number 1,2, 181–184 FEBS 09586 April 1991

ADONIS 001457939100296X

Sequence-specific binding of the N-terminal three-finger fragment of *Xenopus* transcription factor IIIA to the internal control region of a 5S RNA gene

Jørgen Holst Christensen, Peter Kamp Hansen, Ove Lillelund and Hans Christian Thøgersen

Protein Engineering Research Centre, Laboratory of Gene Expression, University of Aarhus, Gustav Wieds Vej 10, DK-8000 Aarhus C, Denmark

Received 5 February 1991

An N-terminal fragment of *Xenopus* TFIIIA, containing domains 1–3, (TF 3), was expressed in *E. coli*. High yields of recombinant zinc finger protein was isolated, and its DNA binding activity for the internal control region (ICR) of the *Xenopus* 5S RNA gene, was demonstrated by band-shift experiments and DNase I footprinting analysis. TF 3 protects 20 bp of ICR against DNase I digestion. The limits of protection are from +77 to +96 on both coding and noncoding strand. This protection pattern is identical to the protection pattern obtained with TFIIIA in the overlapping region, showing that the 3-finger fragment accounts fully for the protein–DNA interactions in TFIIIA–5S RNA gene over this region.

Xenopus; Transcription factor IIIA; Zinc finger; Footprinting; Domain structure; Domain cooperativity

1. INTRODUCTION

TFIIIA, a 40 kDa transcription factor, required for correct transcription of *Xenopus* 5S RNA genes [1], forms a complex with the 5S RNA gene and at least 2 other factors [2,3]. This complex is recognized by RNA polymerase III and promotes the polymerase to initiate transcription of 5S DNA. Proteolytic studies show [4] that a 10 kDa C-terminal fragment of TFIIIA is required for transcription, whereas a 30 kDa N-terminal fragment is sufficient for specific DNA binding. TFIIIA binds to a 50 bp internal control region (ICR) of 5S DNA, which has been mapped in several footprinting experiments [5–7] to about +43 to +96 of the 5S RNA gene.

The classical (Cys_2His_2) zinc finger (reviews [8,9]) was discovered by Miller et al. [10] as 9 mutually homologous segments, each of about 30 residues, in the DNA-binding fragment of TFIIIA. Zinc finger domains were later shown to be present in a multitude of known or presumed nucleic acid binding proteins [11]. More recently, structures of 3 representatives from this class of domains were elucidated by 2D-NMR protein structure analysis. The 3 known classical single-finger structures, Xfin (finger-31) [12], ADR1 (finger-2) [13], and human enhancer binding protein (finger-2) [14] are all quite similar in overall structure, but differ in structural detail [14], as would be expected for different members from a single class of domains. The structural features of each zinc finger defining its sequence preference in interacting with DNA are not yet known, and similarly, the cooperation within and between clusters of zinc fingers in making up highly sequence-specific binding modules remains to be described in structural terms.

In order to study the protein–DNA interactions important for specific binding of TFIIIA to 5S RNA genes at biochemical and structural levels, we have produced a recombinant N-terminal fragment of TFIIIA, containing domain 1–3 (TF 3). Here, we demonstrate by a gel retardation assay that TF 3 can form a specific complex with the ICR and present footprint data to show that the TF 3 protein binds to the 3′-end of the ICR, producing a DNase I footprint extending 20 bp into the ICR which closely corresponds to that of TFIIIA in this region.

2. MATERIALS AND METHODS

A 1.5 kb fragment of a TFIIIA (*Xenopus*) cDNA clone [15], a gift from Dr. D.D. Brown, was modified with appropriate oligonucleotide primers by PCR, in order to produce a sequence coding for a factor Xa-cleavable hybrid protein [16], consisting of an N-terminal hexahistidine segment fused to residues 1–99 of TFIIIA via the factor Xa recognition sequence (Fig. 1). This construct was cloned into the *Nde*I–*Hin*dIII-site of expression vector pT7-PL, generated by replacing the *Nde*I–*Eco*RI-fragment of pRK172 [17] with a polylinker region containing a *Hin*dIII-site. The resulting expression plasmid, pT7-TF 3, was used to transform BL 21 cells. At a cell density of 0.6 (A_{600}), expression was induced by addition of 5–10-fold excess of λ-CE6 phage [18], containing the gene coding for T7 RNA polymerase. Three to four hours after induction the cells were harvested by centrifugation. The recombinant hybrid protein was extracted from inclusion bodies and single-step affinity purified on an NTA-column [19] before releasing the authentic TF 3 polypeptide by cleavage with factor Xa [16]. Full details of TF 3 purification and metal reconstitu-

Correspondence address: H.C. Thøgersen, Protein Engineering Research Centre, Laboratory of Gene Expression, University of Aarhus, Gustav Wieds Vej 10, DK-8000 Aarhus C, Denmark

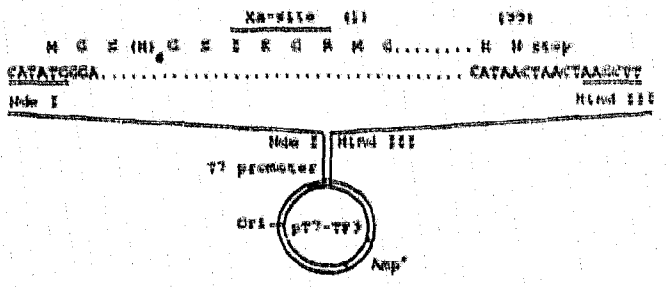


Fig. 1. Construction of pT7-TF3. A 342 bp fragment of the modified TFIIIA cDNA sequence was inserted into the *Nde*I-*Hin*dIII-site of expression vector pT7-PL, as shown. Parts of the nucleotide sequence and the predicted amino acid sequence of TF 3 are shown. The T7 RNA polymerase promoter site (black box) and the β-lactamase gene (open box) are indicated.

tion will be published separately. Protein concentrations were estimated by amino acid analysis.

Plasmid pXbs I, containing a somatic 5S RNA gene from (*Xenopus borealis*) [20], was a gift from Dr. D.D. Brown. A 107 bp *Eco*RV-*Dde*I (filled-in) fragment of pXbs I (corresponding to 5S RNA nucleotides 34 through 120 plus 20 nucleotides of the 3'-non-transcribed region) was excised from pXbs I and recloned in *Sma*I-cut pUC10. From this plasmid (pUC(ICR)), the 128 bp fragment used in these experiments can be cut out using *Bam*HI and *Eco*RI. The coding and the noncoding strand were selectively 3'-labelled, using reverse transcriptase by sequential filling-in reactions at restricted ends with [α-^{32}P]dATP and dNTP. The labelled DNA fragments were isolated after electrophoresis on 1% agarose gels.

TFIIIA was transferred from 7S RNP particles (a gift from Dr. L. Farrell [11]) to DNA as described [17]. Formation of a TF 3-DNA complex was demonstrated by non-denaturing polyacrylamide gel electrophoresis in 90 mM Tris-borate buffer (pH 8.2). For DNase I footprinting analysis, about 0.3 pmol of DNA was mixed with 1 pmol TFIIIA or 0.4–0.5 pmol TF 3 in 180 μl binding buffer (20 mM Tris-HCl, pH 7.5, 70 mM KCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 20 μM zinc acetate, 0.1% nonionic detergent NP-40, and 6% glycerol) and incubated (1 h, 20°C). Parallel experiments were performed with protein-free DNA samples in binding buffer. Samples were then incubated at 20°C with 1 unit DNase I (Promega) fot 60 s, and digestion was terminated by addition of stop solution, 100 μl 1% SDS, 5 mM EDTA and 100 μg/ml sonicated carrier DNA. The samples were extracted with phenol/chloroform, precipitated with ethanol and analyzed by electrophoresis on 10% denaturing acrylamide gels. Sequence assignment of digested products on the autoradiograms (Fuji RX-100 film) was deduced from G+A tracks generated by formic acid-piperidine treatment of DNA.

3. RESULTS AND DISCUSSION

TS 3 was produced in large amounts by specific proteolytic cleavage of a recombinant cleavable hybrid protein made in *E. coli* (Fig. 1). The purified authentic product (Fig. 2) migrates on an SDS-polyacrylamide gel (18%) at an apparent molecular weight of about 13 kDa (calculated, 11.5 kDa). Unique binding of the purified recombinant TF 3 protein to a ^{32}P-labelled DNA fragment containing a fragment of the 5S RNA gene was demonstrated by bandshift analysis (Fig. 3). By titration of fixed amounts of DNA with increasing amounts of recombinant TF 3 protein, a gradual accumulation of a slower discrete band is observed. At limiting pro-

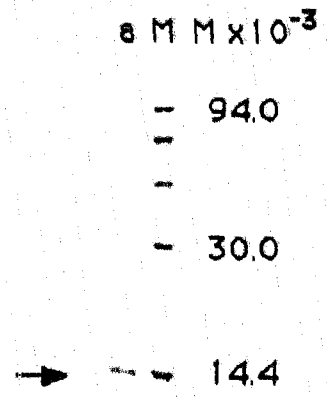


Fig. 2. Expression of TF 3 in *E. coli*. The purified recombinant protein was analysed by 18% SDS-polyacrylamide gel electrophoresis and visualized with Coomassie brilliant blue. Lane (a) purified authentic TF 3 protein and lane (M) protein marker (94, 68, 43, 30, 20 and 14.4 kDa).

tein concentrations, 2 different bands are observed; one corresponding to free DNA and a slower band corresponding to protein-DNA complex. By further increasing the protein concentration only the latter band is observed. These experiments indicate that the recombinant TF 3 protein binds in a stoichiometric and unique fashion to the 5S RNA gene.

The concentration of magnesium ions in the binding buffer is crucial for observing binding of TF 3 to the ICR by bandshift and DNase I footprinting experiments. At the magnesium ion level optimal for binding of TFIIIA (5 mM) no TF 3 binding is observed. In contrast, complete and stoichiometric binding of TF 3 to

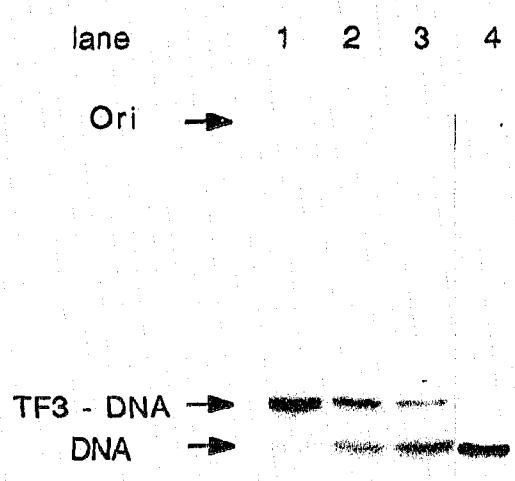
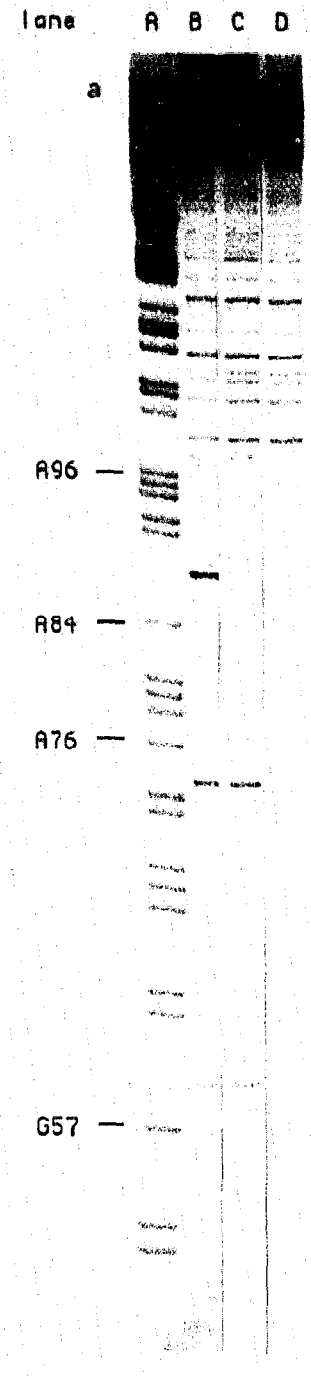


Fig. 3. Binding of TF 3 to the 5S RNA gene. Electrophoretic analysis on a nondenaturing 7.5% polyacrylamide gel of (lanes 1–3) [^{32}P]DNA (approx. 3 pmol) mixed with 4 pmol, 1.8 pmol and 0.4 pmol of TF 3, respectively, and (lane 4) named [^{32}P]DNA.

DNA is achieved at the 1 mM magnesium level. The results of Vrana et al. [18] indicating that N-terminal fragments of TFIIIA shorter than 5 fingers fail to bind, may readily be understood in view of our present result since their assay buffer contained magnesium ions at 5 mM.

Our DNase I footprinting analyses (Fig. 4) show that TF 3 protects a 20 bp DNA region at the 3'-end of the ICR, extending from position +77 to +96 on both strands. Furthermore, the footprints of TF 3 and TFIIIA are identical in this region, showing that all protein-DNA interactions in this region in the TFIIIA-5S RNA gene complex are completely accounted for by the N-terminal 3 zinc fingers of TFIIIA. Footprinting studies



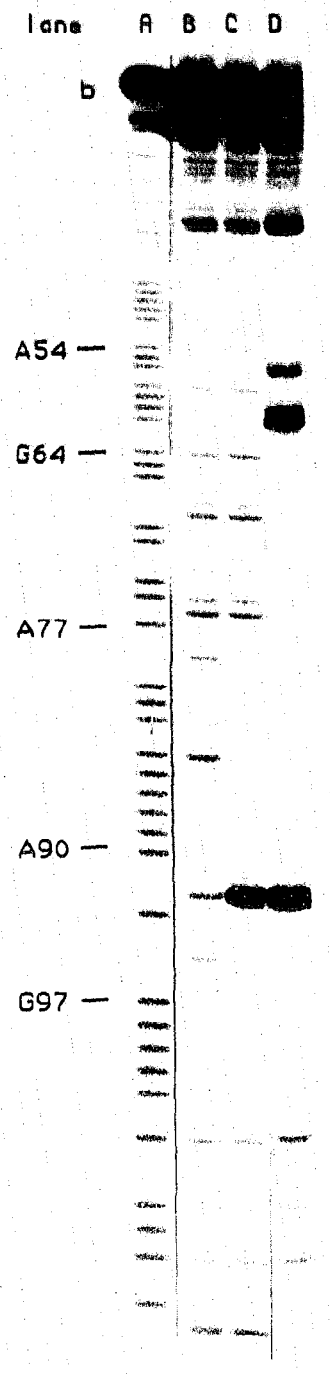


Fig. 4. DNase I digestions of the TF 3- and TFIIIA-5S RNA gene complexes. Autoradiographs of the digestion pattern of (a) the coding strand and (b) the noncoding strand. [^{32}P]DNA was digested at a constant DNase I concentration as (lane B) naked DNA, (lane C) TF 3–DNA complex and (lane D) TFIIIA–DNA complex. Purine tracks are shown in lanes A; numbers refer to sequence positions in the 5S RNA gene.

with a deletion mutant of TFIIIA (TFIIIA (Δ finger-4)) [19], lacking the finger-4, indicate that this mutant protects ICR against DNase I digestion from position +78 to +96. The extra nucleotide protected against digestion with this mutant (bp +78), compared to the footprint we obtain with TF 3, could be due to different Mg^{2+}-levels, and/or to the presence of the C-terminal tail of zinc fingers (domains 5–9), which although not participating in DNA binding, may slightly change the accessibility of the DNA at the 5'-end of the complex. DNase I footprinting experiments performed in wheat germ extracts [18] indicate, that fingers 1–5 of TFIIIA protect about 34 bp of ICR (namely +63 to +96). We have confirmed and extended this result using pure recombinant 5-finger fragment of TFIIIA, TF 5 (Kamp Hansen et al., manuscript in preparation). By subtraction, fingers 4 and 5 of TFIIIA must therefore account for protein-DNA interactions extending along 14 bp. Allowing for the DNase I footprint to extend 1–2 bp beyond either end of the actual binding region for steric reasons, the segments that actually interact with TF 3 and TF 5 are +78/+79 to +94/+95 (16–18 bp) and +64/+65 to +94/+95 (30–32 bp), respectively.

The data presented here, combined with those of Vrana et al. [22] and Hanas et al. [23] contribute to the description of the cooperative behaviour of the 9 zinc fingers in binding of TFIIIA to the 5S RNA gene. Fingers 1–3 (or perhaps an even shorter segment) by themselves, or as part of the TFIIIA (Δfinger-4) deletion mutant, bind specifically to a 16–18 bp segment at the 3'-end of the ICR, precisely as they do in the TFIIIA-DNA complex. The near-identity of the footprints generated by our TF 3 protein and by TFIIIA (Δfinger-4) shows that the absence of finger-4 completely prevents fingers 5–9 from binding to DNA. The results reported by Vrana et al. on the DNA-binding activities of TFIIIA, deleted to various extents from either end, show that truncation of finger-9 apparently also prevents binding of fingers 8, 7 and 6, and furthermore show that disruption of finger 1 essentially abolishes correct binding.

In summary, the binding of TFIIIA to the 5S RNA gene is a strongly cooperative and polarized process, in which the 3 N-terminal fingers must initiate the binding process at the 3'-end of the ICR, which then allows fingers 4 and 5 to bind, in turn, a prerequisite for the strongly cooperative binding of the finger 6–9 cluster of zinc fingers.

Acknowledgements: This work was supported with grants from the NOVO, the Aarhus University and the Carlsberg Foundations, from the Danish Natural Science Research Council, the Danish Biotechnology Research Centre Programme and from the Human Frontiers of Science Programme.

REFERENCES

[1] Engelke, D.R., Ng, S.-Y., Shastry, B.S. and Roeder, R.G. (1980) Cell 19, 717–728.
[2] Segall, J., Matsui, T. and Roeder, R.G. (1980) J. Biol. Chem. 255, 11986–11991.
[3] Bieker, J.J., Martin, P.L. and Roeder, R.G. (1985) Cell 40, 119–127.
[4] Smith, D.R., Jackson, T.J. and Brown, D.D. (1984) Cell 37, 645–652.
[5] Bogenhagen, D.F., Sakonju, S. and Brown, D.D. (1980) Cell 19, 27–35.
[6] Sakonju, S. and Brown, D.D. (1982) Cell 31, 395–405.
[7] Rhodes, D. (1985) EMBO J. 4, 3473–3482.
[8] Klug, A. and Rhodes, D. (1987) Trends Biochem. Sci. 12, 464–469.
[9] Evans, R.M. and Hollenberg, S.M. (1988) Cell 52, 1–3.
[10] Miller, J., McLachlan, A.D. and Klug, A. (1985) EMBO J. 4, 1609–1614.
[11] Gibson, T.J., Postma, J.P.M., Brown, R.S. and Argos, P. (1988) Protein Eng. 2, 209–219.
[12] Lee, M.S., Gippert, G.P., Soman, K.V., Case, D.A. and Wright, P.E. (1989) Science 245, 635–637.
[13] Klevit, R.E., Herriot, J.R. and Horvadt, S.J. (1990) Proteins: Struct. Funct. Genet. 7, 215–226.
[14] Omichinski, J.G., Clore, M., Appelle, E., Sakaguchi, K. and Gronenborn, A.M. (1990) Biochemistry 29, 9324–9334.
[15] Ginsberg, A.M., King, B.O. and Roeder, R.G. (1984) Cell 39, 479–489.
[16] Nagai, K. and Thøgersen, H.C. (1987) Meth. Enzymol. 154, 461–481.
[17] McLeod, M., Stein, M. and Beach, D. (1987) EMBO J. 6, 729–736.
[18] Studier, W. and Moffatt, B.A. (1986) J. Mol. Biol. 189, 113–130.
[19] Hochuli, E., Döbeli, H. and Schacher, A.J. (1987) Chromosoma 411, 177–184.
[20] Peterson, R.C., Doering, J.L. and Brown, D.D. (1980) Cell 20, 131–141.
[21] Pelham, H.R.B. and Brown, D.D. (1980) Proc. Natl. Acad. Sci. USA 77, 4170–4174.
[22] Vrana, K.E., Churchill, M.E.A., Tullius, T.D., Brown, D.D. (1988) Mol. Cell. Biol. 8, 1684–1696.
[23] Hanas, J.S., Littell, R.M., Gaskins, C.J. and Zebrowski, R. (1989) Nucleic Acids Res. 17, 9861–9870.