Absence of substantial bending in *Xenopus laevis* transcription factor IIIA–DNA complexes

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ABSTRACT

The extent and location of DNA-bending induced in the *Xenopus laevis* transcription factor IIIA-oocyte 5S RNA gene complex was determined by the gel retardation method. The electrophoretic mobilities of TFIIIA complexed with restriction fragments of 160, 177, 282 and 300 bp that contain the sequence of the major oocyte 5S RNA gene were compared. In these fragments the 120-bp gene is positioned either in the middle or at the end. Minor differences in the mobility of the complexes indicate that the degree of DNA bending is only slight. To determine the bending angle more precisely, a bending vector system, pBend3, was used to examine the complex of TFIIIA with the internal control region (ICR) of the 5S RNA gene. A 61-bp synthetic duplex corresponding to the ICR sequence was cloned into pBend3. Duplicated circular permuted restriction sites allow several 186-bp fragments to be generated in which the position of the ICR can be varied. Gel retardation of TFIIIA-DNA complexes with the ICR sequence contained in pBend3 indicates a bending angle of only 30 degrees and shows that interaction in the ICR could account for all of the bending found in the complete oocyte 5S RNA gene.

INTRODUCTION

TFIIIA is one of several protein factors necessary for the regulation of transcription of the 5S ribosomal RNA genes in previtellogenic *Xenopus* oocytes. Using cloned *Xenopus* genes there have been many DNA footprinting studies that examine the interaction between TFIIIA and the 60-bp ICR (1–5). However, none of these cloned genes are exactly identical to the sequence of the major oocyte 5S RNA actually expressed *in vivo*. TFIIIA contains nine zinc fingers (6–7) that allow the protein to bind in an extended fashion (8) to the ICR of the *Xenopus* 5S RNA gene. Large amounts of a stable bimolecular complex occur in the oocyte whose components are TFIIIA and 5S RNA (9). The RNA regions that are involved in contacts with TFIIIA have been identified (10–13) but the actual mode of binding in the RNA-protein complex remains unknown. It is an intriguing structural problem of how a single protein is able to recognize and bind extensively in a sequence specific manner to both RNA and DNA. An possible explanation comes from circular dichroism measurements which indicate that the ICR sequence can adopt a double-helical conformation that appears to be intermediate between A-form and B-form (14–15).

The electrophoretic mobility of a complex containing a protein-induced DNA bend is diminished according to the position of the binding site in the DNA fragment and also the degree of bending present (16). In order to detect whether such changes occur during regulation of the SS RNA gene, we have investigated to what degree of DNA-bending occurs in TFIIIA-DNA complexes. At the present time TFIIIA is unprecedented in the large size of its DNA interaction. It would seem entirely reasonable to expect a considerable change in the conformation of the DNA involved in binding to TFIIIA.

In this study the overall DNA-bending is estimated by the gel retardation technique. Stoichiometric amounts of purified TFIIIA protein were added to restriction fragments containing the *X. laevis* major 5S RNA oocyte gene (17) or to a synthetic 61-bp ICR sequence in fragments that were derived from a bending vector, pBend3 (18–19). 186-bp fragments of identical length, but in which the ICR is positioned close to the end or in the center are conveniently generated from pBend3 by the use of single restriction endonucleases.

After this work was completed a report appeared (20) in which evidence is presented for DNA-bending in a complex between TFIIIA and 550-bp fragments that contain the 3' two-thirds of the *X. borealis* somatic 5S RNA gene. An assessment or comparison with these results is difficult since the conformational change induced by TFIIIA in this heterologous complex was not estimated by the authors or related to the degree of bending induced by another DNA-binding protein.

It should be pointed out that the nucleotide sequence of the *X. borealis* somatic gene differs at seven positions from the gene which is the natural target for TFIIIA. Such slight differences are found to significantly alter the fine structure of the TFIIIA-ICR interaction as judged, for example, by DNA footprinting.

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In order to avoid the necessity for extrapolation we have deliberately chosen to study the homologous complex formed between TFIIIA and the X. laevis 5S RNA sequence that is found in large amounts in the previtellogenic oocyte.

MATERIALS AND METHODS

Purification of TFIIIA from Xenopus laevis oocytes

7S particles were prepared from ovaries of immature X. laevis as described in (13) except that a gel filtration was carried out using Sephacryl S-300HR and final concentration was by centrifugation in a Centriprep 30 unit (Amicon). 7S particles were dissociated by the addition of 1 M NaCl and TFIIIA was differentially precipitated with an equal volume of 60% saturated (NH₄)₂SO₄, 1 M NaCl and recovered by centrifugation at 15,000 g for 15 min. at 4°C. The pellet was redissolved in 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM DTT, 2 mM benzamidine-HCl, 1 mM NaN₃ and 50% glycerol and stored under nitrogen gas at -20°C. The concentration of TFIIIA was determined by the protein assay (Bio-Rad) using BSA as a standard and a conversion factor of 0.62 ± 0.06. This factor was found by comparison with the concentration derived by micro-Kjeldahl analysis of the organic nitrogen in TFIIIA (22).

Construction of bending vector pB-ICR

Two complementary 61-mer oligonucleotides whose sequences correspond to position 40 to 100 of the X. laevis oocyte 5S RNA gene were synthesized (their sequences are shown in Figure 1A, top). Oligonucleotides were extracted sequentially with phenol, phenol/chloroform and chloroform, lyophilized in a vacuum centrifuge and taken up in water. An aliquot of each sample was purified on a 25% polyacrylamide gel (acrylamide/bisacrylamide 29:1) containing Tris-borate/EDTA (23). Oligonucleotides were extracted from the gel slices by shaking over night at room temperature in 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, pH 7.5, 100 mM NaCl, 0.1% SDS and were annealed in the same buffer by heating in a 95°C water bath for 5 min. followed by cooling to room temperature in four hours. The DNA duplex was extracted with phenol/chloroform and precipitated by adding 3 vols. of ethanol and 300 mM NaCl. A 10-fold molar excess of the duplex was mixed with pBend3 DNA which had been linearized with restriction endonuclease Sal1, and filled in with DNA-polymerase

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![Diagram](https://via.placeholder.com/150)

Figure 1: A: pB-ICR was constructed by insertion of a 61-bp synthetic DNA fragment (shown on top) comprising the internal control region (ICR) of the X. laevis oocyte 5S RNA gene into the Sal1 site of the pBend3 vector (see Material and Methods). The direction of transcription of the bottom strand is indicated by the arrow. DNA fragments of 186 bp length which differ in the positioning of the ICR, are generated from pB-ICR by digestion with the restriction endonucleases Mlu1 (M), EcoRV (E) or BamH1 (B). B: DNA-fragments of similar size (177 and 160 bp) and of larger size (282 and 300 bp) which contain the oocyte 5S RNA gene were obtained from pXlo-wt (17) by digestion with Pvu1 and EcoR1 (P/E), Mac3 and Hpa2 (M/H), Eae1 (Ea) or Hind3 and EcoR1 (H/E).
The sequence of the EcoRI/Hind3 fragment containing a XbaI and a SalI cloning site as well as seventeen duplicated circular permuted restriction sites has been published recently (19). The construction of pBend3 will be described in detail elsewhere. After transformation of competent Escherichia coli DH5α cells, miniprep DNA of a dozen transformants was prepared and screened for the presence of the 61-bp insert by restriction mapping with EcoRI, Hind3, XbaI, MluI, EcoRV and BamHI. The ICR orientation and sequence of one of the positive clones was confirmed (23).

**Binding of TFIIIA to DNA fragments**

PBl-ICR and pXlo-wt (17) was prepared by centrifugation on CsCl gradients (24). Plasmid DNA was digested with suitable restriction endonucleases (see Results). The enzymes were removed by precipitation in the presence of 2.5 M NH₄Cl and the DNA was recovered from the supernatant with two volumes of ethanol. The pellet was washed with 80% ethanol, dried in a vacuum centrifuge, dissolved in a small amount of water and stored at -70°C. TFIIIA was bound to digested DNA essentially as described in (25) in 20 mM Tris-HCl pH 7.5, 70 mM NH₄Cl, 7 mM MgCl₂, 1 mM DTT, 10 mM ZnCl₂, 10% glycerol. A 10 μl reaction mix was prepared at room temperature by combining in the following order: 1.5 μl 5× binding buffer, 3 μl DNA (3 μg of digested plasmid), 3.5 μl water, 2 μl TFIIIA in 1× binding buffer. The TFIIIA stock (5 mg/ml protein) was diluted 1/100 immediately prior to use. The condition gives rise to the formation of mainly complex with some amount of unbound DNA fragment. No complexes of higher stoichiometries are observed. After incubation at room temperature for 10 min. the samples were loaded directly onto an 8% polyacrylamide gel (25) without the addition of tracking dyes. Electrophoresis was at room temperature at 200 V for 5 hours. The gel was immersed in Tris-acetate buffer (24) with 1 μg/ml ethidium bromide to stain the DNA for visualization under UV light.

**RESULTS**

The electrophoretic behavior of various TFIIIA-DNA complexes is shown in Figures 2 and 3. It is expected that the gel mobility of the complexes will be affected by the precise location of any DNA bending present in them. The greatest retardation will be observed when the bend is at the center of the DNA fragment. This can be measured conveniently by comparison with a complex in which the protein binds close to the end of the DNA fragment. An obvious difference can be seen in Figures 2 and 3 in the

![Figure 2](image-url)

Figure 2: Formation of complexes of X. laevis TFIIIA with DNA fragments generated from the bending vector pB-ICR. DNA fragments and purified TFIIIA were mixed as described in Material and Methods, and were loaded onto an 8% polyacrylamide gel. Unbound DNA-fragments containing a protein binding site are marked by •; complexes are marked by ▲. Formation of complexes of X. laevis TFIIIA with DNA fragments generated from the bending vector pB-ICR. M: DNA digested with MluI, E: EcoRV, B: BamHI. a: no TFIIIA; b: 6 μg/ml TFIIIA; c: 60 μg/ml TFIIIA; CRP: Complexes between the cyclic AMP receptor protein (CRP) and the lac promoter sequences contained in pBend2 (18,19). m: Marker DNA fragments from a Hae3 digest of φX174 DNA.
The mobility of the CRP-DNA complex in an 8% polyacrylamide gel at different positions as shown in Figure 1B (the fragments of pXlo-wt containing the complete oocyte 5S RNA gene X. laevis experiments are shown in Table 1. In agreement with previous results (19) we observe a bend of about 90° induced by CRP binding to a pB-ICR-derived BamHl-fragment of similar size (Figure 2, lanes B-a,b,c) which does not contain the ICR sequence. Surprisingly, complexes of TFIIIA with the smaller DNA fragments 177 bp, 160 bp (Figure 3, lanes 5 and 6) have quite similar gel mobilities to those with 300 bp and 282 bp (lanes 7 and 8). Anomalous electrophoretic behavior implies that some modification of their overall shape has taken place. It is possible that the larger TFIIIA-DNA complexes have become somewhat more compact through condensation of the DNA. As expected, all of the unbound restriction fragments are seen to migrate in the gel according to their molecular weights which indicates the absence of any inherent bend in the free DNA.

The interaction between TFIIIA and the isolated 61-bp ICR sequence was also evaluated by the gel retardation technique described above. The ICR was inserted into the SalI cloning site of the pBend3 vector system and the resulting vector was called pB-ICR (Figure 1A). Digestion of the pB-ICR with MluI or BamHI generates two 186-bp fragments in which the ICR is positioned close to either end. Similarly, a 186-bp restriction fragment with the ICR located in the middle is produced upon digestion with EcoRV. Conditions for binding stoichiometric amounts of TFIIIA to these fragments are demonstrated in Figure 2. Binding is specific, as indicated by the failure of TFIIIA to bind to a pB-ICR-derived BamHI-fragment of similar size (Figure 2, lanes B-a,b,c) which does not contain the ICR sequence. In contrast to the high degree of bending induced by CRP, only a slight bending of the ICR-containing fragments is detected in the presence of TFIIIA. The small mobility difference observed between the terminally and the centrally located ICR, represents a bend of about 30°. In Figure 3 (lanes 3-6) it can be seen that the gel mobilities of TFIIIA-DNA complexes containing either the ICR or the complete oocyte 5S RNA gene are virtually identical. Again only small differences are observed between TFIIIA complexes corresponding to the terminally and the centrally located ICR sequence in 186-bp fragments.

**DISCUSSION**

The extensive interaction between nine zinc fingers of TFIIIA and the 60-bp internal promoter of the 5S RNA gene has the potential for considerable conformational changes to occur in both protein and the DNA binding site. Indeed protein-induced alteration of the double helix structure could enhance the rate of transcription and replication of DNA. In contrast to the CRP-DNA complexes described above the mobility of complexes between the cyclic AMP receptor protein (CRP) from E. coli and 163-bp DNA fragments. These contain a 38-bp sequence of the lac promoter CRP-binding site and have been produced by digestion of pBend2 as described in (19).

The bending angle $\alpha$ is defined as the angle by which a segment of the DNA departs from linearity, for example, 0° is the value for a straight duplex. $\alpha$ can be estimated from gel mobilities (19) using the relationship $\mu M/\mu E = \cos(\alpha/2)$. Here $\mu M$ = mobility of the complex with the protein bound centrally and $\mu E$ = mobility of the complex with the protein bound at the end of a DNA fragment. Values for $\alpha$ obtained from three independent experiments are shown in Table 1. In agreement with previous results (19) we observe a bend of about 90° induced by CRP binding to the lac promoter sequence.

Two restriction fragments were generated from the plasmid pXlo-wt containing the complete X. laevis oocyte 5S RNA gene at different positions as shown in Figure 1B (the fragments of 160 and 177 bp are comparable in size to those (186 bp) discussed below which contain the ICR sequence obtained from pB-ICR). The mobility of the CRP-DNA complex in an 8% polyacrylamide gel is found to be similar to the TFIIIA-DNA complex (Figure 2 and 3) when both proteins bind at the ends of equivalent restriction fragments. However, in contrast to CRP only a small change in gel mobility is seen when TFIIIA binds centrally (Figure 3, lanes 6 and 5). Comparison of the complexes between TFIIIA and larger restriction fragments of 282 bp and 300 bp (Figure 3, lanes 8 and 7) confirms this result. An overall mobility difference consistent with only slight bending of the oocyte 5S RNA gene in complexes with TFIIIA is detected.

### Table 1: Comparison of DNA bending angles induced by CRP and TFIIIA (Values from three independent experiments are shown for each protein)

<table>
<thead>
<tr>
<th>Protein</th>
<th>DNA binding site</th>
<th>Length of DNA fragment</th>
<th>Relative mobility</th>
<th>Apparent bending angle $\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli CRP</td>
<td>CRP binding site of lac promoter</td>
<td>163 bp</td>
<td>0.694</td>
<td>92°</td>
</tr>
<tr>
<td>X. laevis TFIIIA</td>
<td>ICR of 5S RNA gene in X. laevis oocyte</td>
<td>186 bp</td>
<td>0.974</td>
<td>26°</td>
</tr>
</tbody>
</table>

In Figure 3: TFIIIA-DNA complexes analyzed on an 8% polyacrylamide gel. The gel mobilities of TFIIIA-DNA complexes containing either the ICR or the complete oocyte 5S RNA gene are virtually identical. Unbound DNA-fragments containing a protein binding site are marked by $*$; complexes are marked by A. Lanes 1 and 2: CRP with fragments from pBend2; Lanes 3 to 8: TFIIIA with fragments from pB-ICR containing the ICR sequence (in lanes 3 and 4) or with fragments from pXlo-wt carrying the oocyte 5S RNA gene (in lanes 5 to 8). No TFIIIA was added in lane 9. The DNA-fragments were generated with the following restriction enzymes (see also Figure 1): lane 1, Mlu1 (163 bp), lane 2, EcoRV (163 bp), lane 3, Mlu1 (186 bp), lane 4, EcoRV (186 bp), lane 5, EcoRI and Hinc3 (177 bp), lane 6, EcoRI and EcoR1 (160 bp), lane 7, PvuI and EcoR1 (300 bp), lanes 8 and 9, Mac3 and Hpa2 (282 bp).
lac promoter complex we find that the overall site-specific bending of DNA by TFIIIA is in fact only slight as detected by gel retardation.

The degree of DNA-bending present in TFIIIA-5S RNA gene complexes observed amounts to about 30° in contrast to an about 90° bend induced by CRP (19,26) in restriction fragments of comparable size. This result appears to conflict with a similar study of DNA-bending in a complex consisting of TFIIIA and 550-bp fragments containing a 5'-truncated *X. borealis* somatic 5S gene (20). Although the bending angle was not estimated, it was concluded that the TFIIIA/DNA complex may have a compact, globular shape caused by DNA bending. Support for this proposal is provided by a high-dose electron microscopy analysis of phosphorus in the complex that attempts to describe the path of the 5S RNA gene (27).

Our results clearly demonstrate the absence of substantial DNA-bending in both the oocyte 5S RNA gene and in the isolated ICR in the presence of TFIIIA. All of the bending detected can be attributed to the ICR sequence. It has previously been found that TFIIIA produces no appreciable change in the structure of 5S RNA (10–13) in the 7S particle. Furthermore the small differences in gel mobility of TFIIIA-DNA complexes are evidence that DNA remains largely unaffected. This is entirely consistent with an extended mode of binding as in the elongated model of the 7S particle determined by neutron diffraction (28).

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REFERENCES
