Binding of Yeast TFIIIC to tRNA Gene Bipartite Internal Promoters:
Analysis of Physical Effects on the Intervening DNA

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Complexes between transcription factor TFIIIC and eukaryotic tRNA gene internal promoter A and B boxes are unusual in that the binding to the two distinct sites tolerates considerable variation in both distance and helical orientation between the sites. Electrophoretic mobility of *Saccharomyces cerevisiae* TFIIIC complexes with circularly permuted tRNA gene fragments and sensitivity of the complexes to a single stranded-specific reagent, potassium permanganate, indicated that no significant bend or distortion was introduced into the DNA by simultaneous binding to both internal promoters. These data support a model in which variability in the relative positions of the two binding sites is compensated by flexibility in the structure of TFIIIC. © 1991 Academic Press, Inc.

The initial transcriptional activation of tRNA genes in eukaryotes involves the binding of transcription factor IIIC (TFIIIC) to distinct A box and B box internal promoters, followed by binding of transcription factor IIIB (TFIIIB) and RNA polymerase III (pol III) to the transcription start site and upstream region (1,2). Several lines of evidence suggest that yeast TFIIIC is a multipolypeptide complex (3-5) in which different regions or subunits of a single molecule simultaneously interact with different regions of a tRNA gene, including the A and B boxes (4). Although in higher eukaryotes TFIIIC has been chromatographically separated into more than one component (6,7), the yeast bipartite binding activity is seen as a single entity even after TFIIIC has been purified on a DNA affinity resin containing only B box sequences (1,5, this report). This is further supported by the strong dependence of A box interactions on simultaneous binding of yeast TFIIIC to the B box (8).

TFIIIC binds optimally to genes having A-B distances of 30-60 bp, with a minimum of 21 bp required for accurate initiation of transcription (9). Surprisingly, there is a lack of preferred helical orientation between the A and B boxes. Communication between protein components bound at distinct sites on DNA has generally been found to require a fixed "sidedness" of the binding sites, even in cases where the DNA bends to correct for distance (10-16). Since TFIIIC binds to both the A and B boxes it might be a very flexible protein complex as has been suggested for the yeast α2 protein (17). Alternatively, the binding of TFIIIC causes the DNA between the A and B boxes to loop or bend in a way that corrects the spatial and helical orientations of the two binding sites.

Examination of the binding of TFIIIC to a tRNALeu3 gene by electron microscopy indicated that the majority of protein-DNA complexes contained a sharp DNA bend at the binding site (18). Recently, however, the opposite conclusion was reached by observing the binding of TFIIIC to various tRNA genes

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using scanning transmission electron microscopy (19). We have previously suggested (20) a bent complex based on anomalies in DNase I sensitivity (12), but in this report we demonstrate the absence of DNA distortions in TFIIIC complexes by two different methods.

**MATERIALS AND METHODS**

**Plasmids and DNA Fragment Preparation** The 695 bp EcoRI-BamHI fragment from pHN915 (23), containing a direct repeat for circular permutation, was subcloned into the EcoRI and BamHI sites of pUC9 to give plasmid pUCHN. Various tRNA- leu(SUP53) gene variants in pUC9 (20) and a tRNA-Tyr(SUP4) gene (24) were cloned into the Xhol site of pUCHN as follows. The SUP53 genes from -60 to +130 were amplified (25) with oligonucleotides that created EcoRI and Clal linker sites at the upstream and downstream termini. A 249 base pair BamHI fragment of the SUP4 tRNA gene (from pC689, ref. 24) was blunted by end filling. Xhol linkers (8mers) were added to all fragments and the fragments were cloned into the unique Xhol site between the pUCHN direct repeats (Figure 1).

Confirmation of orientation and the correct DNA sequences was obtained by DNA sequencing (26). The plasmids constructed contained the wild-type SUP53 gene (pSUP53), a variant with the G at position 19 changed to a C (SUP53(a)), a variant containing a 19 bp deletion in the intervening sequence [SUP53(A19)], a variant containing the 19 bp deletion and the G19 to C change [SUP53(a/A19)] (27,28), and the SUP4 gene (SUP4). tRNA genes used in bending studies are depicted in Fig. 1. 

**Mobility Shift Assays** Preparation of the affinity-purified TFIIIC from S. cerevisiae PP1002 is described in detail elsewhere (20, 30-32). DNA fragments containing tRNA genes were excised from pUCHN with the five restriction endonucleases shown in Fig 1, phosphorylated with [γ-32P]-ATP (6,000 Ci/mmol) (33), and isolated on nondenaturing polyacrylamide gels. As a positive control for our ability to observe protein-induced DNA bending, we also performed bending experiments (not shown) with E. coli CAP protein and the lac promoter from a pHW104 clone (22, protein and DNA kindly provided by M. Gartenburg and M. Crothers). Binding site position-dependent electrophoretic mobility differences were similar to the published values (22).

End-labelled DNA fragments and 0.5 μl of affinity column-purified TFIIIC were incubated for 12 min at room temperature in a final volume of 10 μl of assay buffer [20 mM HEPES (pH 7.9), 70-85 mM (NH4)2SO4, 4 mM MgCl2, 5.5% glycerol, 1 mg/ml BSA, and 0.1 μg Rsal cut pUC9]. Samples were loaded onto 1 cm X 13 cm X 20 cm 4% polyacrylamide gels (acrylamide:bis, 70:1) containing 5% glycerol and 0.25X TBE that had been prerun for 10 min at 100 V at room temperature. Gels were run in 0.25X TBE at 100 V for 15 min, then transferred to 4OC and the voltage increased to 350 V for about 2 h. The gel was dried and exposed to Dupont Cronex film with a Lightening Plus intensifying screen for 12-24 h.

**Footprinting** SUP53 and SUP53(a) were end-labelled on the transcribed strand by cleaving pUCHN derivatives with HindIII and EcoRV, filling in the HindIII end with [α-32P]dATP (3000 Ci/mmol) using the Klenow fragment of DNA polymerase, and then purifying the 365 bp fragment containing the gene on a 7% native gel. Approximately 2 ng of end-labelled DNA (8000 cpm) was used for DNase I footprinting reactions as described (27, 32).

**KMnO4 Analysis** The KMnO4 procedure was based on the method by Borowiec et al. (35), but with modified T7 DNA polymerase (34) reactions (20) to detect modification sites. pUC53 (40 ng) alone or complexed with 3 μl of TFIIIC fraction was incubated for 12 min at room temperature in a final volume of 20 μl containing 30 mM Tris (pH 7.9), 4 mM MgCl2, 130 mM KCl, and 1 μg Rsal cut pUC9. A solution of 200 mM KMnO4 in 20 mM Tris (pH 7.0) was made up immediately before use. KMnO4 was added to the reactions (2-4 μl), incubation continued for 4 min, and the reactions were quenched with 2 μl of 2-mercaptoethanol (14.7 M). Next, 25 ml of water were added and the samples were passed through a 200 μl Sephadex G-50 (Pharmacia) spin column equilibrated with 50 μl water. The DNA was ethanol precipitated with 2.5 volumes of ethanol and 0.1 volumes of 3 M sodium acetate. The DNA was then cleaved with HindIII (primer 2 reactions) or EcoRI (primer 3 reactions). In parallel reactions DNase I was added instead of KMnO4 and primer extension footprinting was carried out as described (20) to confirm occupation by TFIIIC (not shown). Primer 2 hybridized to the transcribed strand at positions -60 to -45 relative to the transcription initiation site (20). Primer 3 hybridized to the non-transcribed strand past the transcription termination site at position +177 to +162 (2). Primer extensions were performed and analyzed as described (2).

**RESULTS AND DISCUSSION**

**Gel Mobility Shift Assays With Circularly Permutated DNA Fragments**

To determine whether the binding of TFIIIC to tRNA genes induces a bend in the DNA, various tRNA genes were cloned into the circular permutation vector pUCHN (Fig. 1). The genes used in the circular
permutation mobility shift assay were the SUP53 gene, the SUP53 gene containing a 19 bp deletion in the intervening sequence [SUP53(Δ19)], a gene identical to SUP53(Δ19) except for a point mutation in the A box that changes G19 to a C [SUP53(a/Δ19)], and the SUP4 gene (see Fig. 1). The 19 bp deletion in SUP53(Δ19) results in an A-B box spacing of 55 bp, which is within the optimal spacing range for TFIIIC binding as defined by Baker et al. (9). The A and B boxes in SUP4 are separated by 48 bp. The circular permutation vector can be cleaved with the restriction enzymes shown in Fig. 1A, yielding 5 fragments of identical size and base pair composition that vary only as to the position of the tRNA gene with respect to the ends of the DNA fragment.

When purified TFIIIC was bound to a radiolabelled DNA fragment containing the SUP53 gene, the protein-DNA complex had a much reduced electrophoretic mobility compared to the free DNA fragment (Fig. 2). The addition of excess unlabelled plasmid DNA containing the SUP53 gene (lane WT), but not vector DNA alone (lane V), resulted in the disappearance of the shifted band. This indicated that the shifted band was a specific complex formed by TFIIIC with the tRNA gene.

Analysis of TFIIIC complexes with circularly permuted DNA fragments are shown in Fig. 3. Very little variation in mobility of the shifted TFIIIC-tRNA gene complexes was seen with the permuted DNA fragments from plasmids containing SUP53 gene (panel A), SUP53(Δ19) (panel B), or SUP4 gene (panel C) genes. In all cases, in which the tRNA gene was located near the end of the fragment (HindIII and EcoRV fragments,) had slightly faster mobilities than with the BstNI, EcoRV, and Nhel fragments. These differences in the mobility shifts were too small (<2 mm difference on migration distances up to 4 cm), but bands became blurred with much longer runs without significantly increasing mobility differences.

This small migration anomaly is not due to TFIIIC bending the DNA to align its contacts with the A and B boxes. This was demonstrated using a SUP53 A box mutation that eliminates stable binding of TFIIIC at the A box while maintaining binding at the B box (Fig. 4, ref. 32). The A box mutation was combined with the Δ19 intron deletion for the mobility shifts because of the higher affinity of TFIIIC for this closer
Figure 2. Gel mobility shift analysis of TFIIIC-tRNA gene complexes. The SUP53 gene end-labelled at the HindIII site in pSUP53 was incubated in the absence (-) or presence (+) of affinity column purified TFIIIC. Binding reactions and electrophoresis of complexes were performed as described in Materials and Methods. Competitor DNA (comp. DNA, 0.2 μg) was plasmid pUC9 (lane V) or pUC9 containing the wild-type SUP53 gene (lane WT). Trace amounts of complexes migrating between the free DNA and TFIIIC shift are at positions consistent with intermediate shifts obtained through partial proteolysis of TFIIIC (32, 38, unpublished observations).

Figure 3. Electrophoretic analysis of circularly permuted fragments in the presence of TFIIIC. Mobility shift assays were performed as described in Materials and Methods with affinity-column-purified TFIIIC and the circularly permuted end-labelled fragments diagrammed in Fig. 1. Circularly permuted, unbound DNA fragments had indistinguishable electrophoretic mobilities (not shown).

spacing of A and B boxes. Analysis of the permuted DNA fragments containing SUP53(A19) and SUP53(a/A19) complexed with TFIIIC showed that very similar shifted patterns were seen with the wild type A box [SUP53(A19)] and the mutated A box [SUP53(a/A19)]. Thus the absence of stable binding of TFIIIC to the A box does not affect the mobilities of the permuted complexes. The cause of these small mobility differences among the complexes is not clear. It is possible that there is an induced bend that depends only on B box binding and does not unwind the DNA sufficiently to cause KMnO4 sensitivity (below). Alternatively, the existence of a large protein complex such as TFIIIC on the middle of a DNA fragment might impede migration of the complex through a gel more than when it is near an end of the fragment. It was not possible to do an analogous experiment with a tRNA gene containing a point mutation in the B box, since such a mutation abolishes stable binding of TFIIIC to the tRNA gene (20, 27).

KMnO4 Analysis of TFIIIC-tRNA Gene Complexes

To determine whether the binding of TFIIIC to tRNA genes might induce melting or unwinding of the DNA helix, TFIIIC-DNA complexes were probed with KMnO4 (35). KMnO4 oxidizes the 5,6 double-bond of pyrimidines preferentially in regions with single-stranded DNA (36). TFIIIC was bound to circular plasmid containing the SUP53 gene, treated with KMnO4, and then primer extension analysis was done using modified T7 polymerase to detect modification sites. Figure 5 shows that TFIIIC binding decreases the overall KMnO4 modification on both strands relative to the reactivity of the naked DNA, but that the pattern of modification sites was exactly the same with and without protein. If the DNA structure was
Figure 4. DNase I footprinting of the SUP53 gene and the SUP53 gene containing an A box mutation. An end-labelled DNA fragment containing the wild-type SUP53 gene (SUP53) or the SUP53 gene containing a point mutation in the A box [SUP53(a)] was incubated without (-) or with (+) TFIIIC and the complexes were treated with DNase I as described in Materials and Methods. The region encoding the primary transcript is represented by the arrow to the right of the panel, with the A and B boxes indicated.

Figure 5. Primer extension analysis of KMnO4-treated TFIIIC-tRNA gene complexes and primer extension footprinting of the SUP53 gene. pUC53 plasmid containing the SUP53 gene was incubated with (+) or without (-) TFIIIC and treated with KMnO4 (+, 20 mM; ++, 40 mM). Primer extension determination of the DNA modification sites was done as described in Materials and Methods. The region encoding the primary transcript is represented by the arrows next to the panels, with the A and B boxes indicated.

distorted or melted, regions of hyperreactivity would be expected (35,37,38). Thus, KMnO4 showed no evidence of distorted DNA structure caused by the binding of TFIIIC to the A and B boxes.

Baker et al. (9) suggested that some sort of distortion of the DNA structure (bending, unwinding) may be necessary to allow TFIIIC to bind with similar binding affinities to A and B boxes separated by various amounts of DNA. Examination of complexes by electron microscopy has given mixed results. One early study showed sharply bent DNA at a globular TFIIIC complex (18). More recently, however, highly purified TFIIIC was shown to be one large globular structure only on tRNA genes with closely spaced A and B internal promoters (19). With increasing distance between the binding sites the complex appeared
as two smaller globular complexes, possibly connected, spaced by an amount that increased with the A-B distance. Only at A-B separations greater than 70 bp was looping of the DNA observed between the binding sites. Very recently, this flexible protein model was supported by two additional findings. Increasing the flexibility of the DNA between the A and B boxes by introducing single-strand nicks does not increase complex formation (41). Also, higher order tRNA gene transcription complexes (that have altered TFIIIC contacts relative to binding of TFIIIC alone) do not have notable KMnO4 sensitivity between the A and B boxes (42).

The results presented here support the view that the multipolypeptide TFIIIC factor has sufficient flexibility to "reach" around the helix to contact the A and B boxes without distorting the DNA helix. Further studies of TFIIIC will have to address the mechanism by which this protein can adapt to varying A-B box distances and helical orientations, and how it can interact with different transcription complexes on the various classes of RNA pol III genes.

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REFERENCES