Binding of Yeast TFIIIC to tRNA Gene Bipartite Internal Promoters:

Analysis of Physical Effects on the Intervening DNA

Claire F. Evans[*] and David R. Engelke

Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109-0606

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 tRNA^{Leu3} 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

---

[*] Present address: Department of Neuropharmacology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037.

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 tRNALeu(SUP53) gene variants in pUC9 (20) and a
tRNATyr(SUP4) gene (24) were cloned into the XhoI site of pUCHN as follows. The SUP53 genes from
-60 to +130 were amplified (25) with oligonucleotides that created EcoRI and ClaI 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. XhoI linkers (8mers) were added to all fragments and the
fragments were cloned into the unique XhoI 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(Δ19)], a variant containing the 19 bp deletion and the G19 to C change [SUP53(a/Δ19)] (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 RsaI 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 pUC9 as carrier. 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 Permuted 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
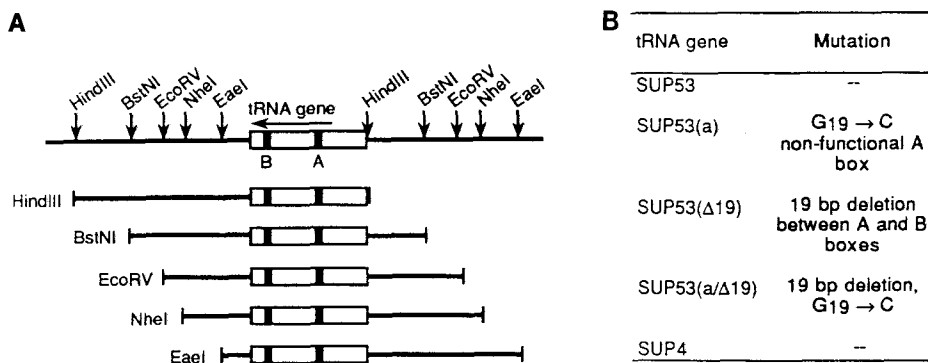
Figure 1. Restriction fragments and tRNA genes used in the circular permutation gel mobility shift assays. A: tRNA genes were cloned into the circular permutation vector pUCHN as described in Materials and Methods. Cleavage of the vector with the indicated set of restriction enzymes produces five fragments 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 fragments. The orientation of the tRNA genes is indicated by the horizontal arrow, with the positions of the A and B boxes marked by black boxes. B: The tRNA genes and gene variants that were cloned into pUCHN are listed.

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 EaeI fragments,) had slightly faster mobilities than with the BstNI, EcoRV, and NheI 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
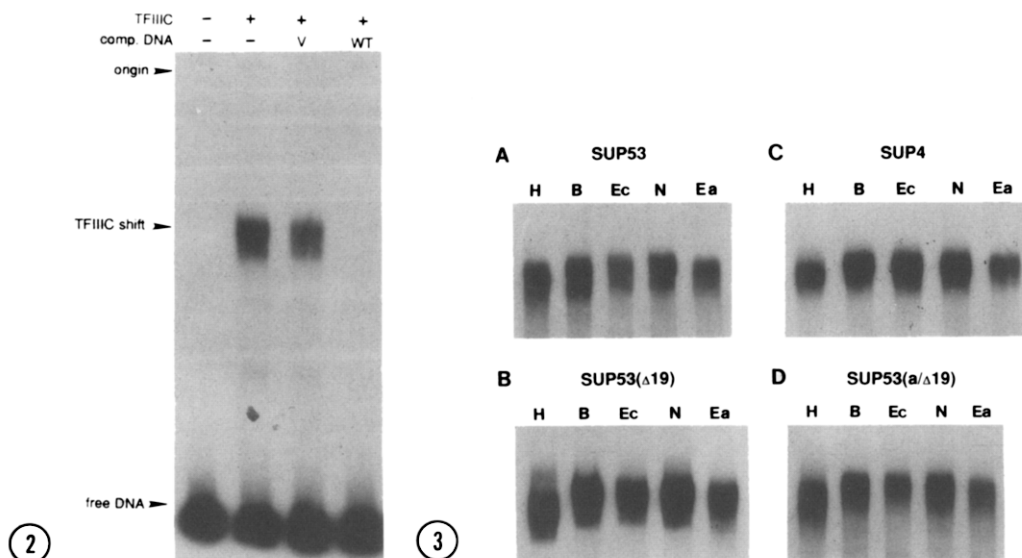
Figure 2. Gel mobility shift analysis of TFIIIC-tRNA gene complexes. The *SUP53* gene end-labelled at the HindIII site in pSUP53 was incubated in the absence (-) or presence (+) of affinity column purified TFIIIC. Binding reactions and electrophoresis of complexes were performed as described in Materials 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*(Δ19) and *SUP53*(a/Δ19) complexed with TFIIIC showed that very similar shifted patterns were seen with the wild type A box [*SUP53*(Δ19)] and the mutated A box [*SUP53*(a/Δ19)]. 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 KMnO$_4$ 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).

KMnO$_4$ 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 KMnO$_4$ (35). KMnO$_4$ 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 KMnO$_4$, and then primer extension analysis was done using modified T7 polymerase to detect modification sites. Figure 5 shows that TFIIIC binding decreases the overall KMnO$_4$ 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
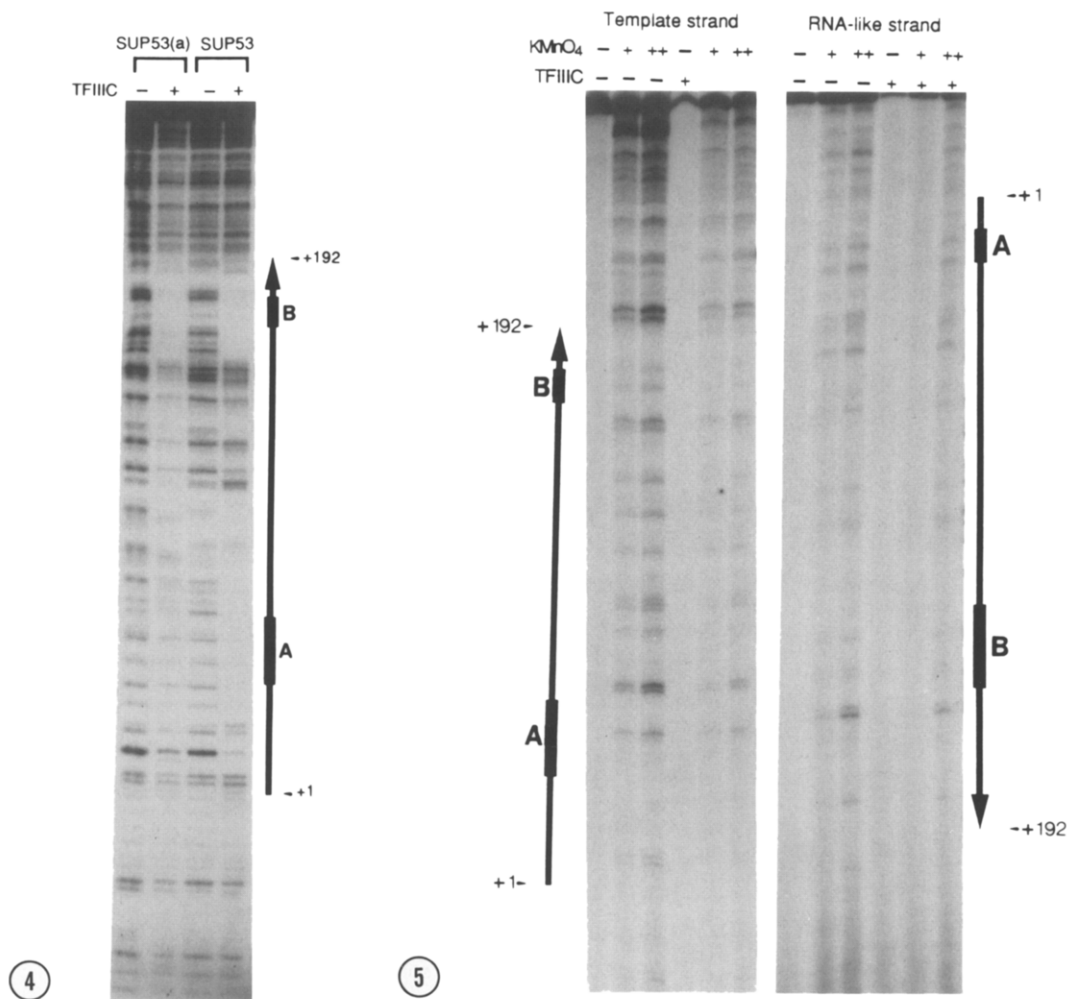
Figure 4. DNase I footprinting of the *SUP53* gene and the *SUP53* gene containing an A box mutation. An end-labelled DNA fragment containing the wild-type *SUP53* gene (*SUP53*) or the *SUP53* gene containing a point mutation in the A box [*SUP53*(a)] was incubated without (-) or with (+) TFIIIC and the complexes were treated wtih 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 KMnO$_4$-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 KMnO$_4$ (+, 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, KMnO$_4$ 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 $KMnO_4$ 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.

REFERENCES

1 Kassavetis,G.A., Riggs,D.L., Negri,R., Nguyen,E.P. L.H. and Geiduschek, E.P. (1989) Mol. Cell. Biol. 9, 2551-2566.
2 Huibregtse,J.M. and Engelke,D.R. (1989) Mol. Cell. Biol. 9, 3244-3252.
3 Gabrielsen,O., Marzouki,N., Ruet,A. Sentenac,A. and Fromageot,P. (1989) J. Biol. Chem. 264, 7505-7511.
4 Bartholomew,B. Kassavetis,G., Braun,B. and Geiduschek,E. (1990) EMBO J. 9, 2197-2205.
5 Parsons,M.C. and Weil,P.A. (1990) J. Biol. Chem. 265, 5095-5103.
6 Yoshinaga,S.K., Boulanger,P.A. and Berk,A.J. (1987) Proc. Natl. Acad. Sci. USA 84, 3585-3589.
7 Ottonelo,S., Rivier,D.H., Doolittle,G.M., Young,L.S. and Sprague,K.U. (1987) EMBO J. 6, 1921-1927.
8 Baker,R., Gabrielsen,O. and Hall,B. (1986) J. Biol. Chem. 261, 5275-5282.
9 Baker,R., Camier,S., Sentenac,A. and Hall,B. (1987) Proc. Natl. Acad. Sci. USA 84, 8768-8772.
10 Bogenhagen,D.F. (1985) J. Biol. Chem. 260, 6466-6471.
11 Takahashi,K., Vigneron,M., Matthes,H., Wildeman,A., Zenke,M. and Chambon,P. (1985) Nature 319, 121-126.
12 Hochschild,A. and Ptashne,M. (1986) Cell 44, 681-687.
13 Dunn,T., Hahn,S., Ogden,S. and Schleif,R. (1984) Proc. Natl. Acad. Sci. 81, 5017-5020.
14 Ross,W. and Landy,A. (1982) Proc. Natl. Acad. Sci. USA 79, 7724-7728.
15 Moitoso de Vargas,L., Pargellis,C.A., Hasan,N.M., Bushman,E.W. and Landy,A. (1988) Cell 54, 923-929.
16 Moitoso de Vargas,L., Kim,S. and Landy,A. (1989) Nature 244, 1457-1461.
17 Sauer,R.T., Smith,D.L. and Johnson,A.D. (1988) Genes Dev. 2, 807-816.
18 Stillman,D.J., Better,M. and Geiduschek,E.P. (1985) J. Mol. Biol. 185, 451-455.
19 Schultz,P., Marzouki,N. Marck,C., Ruet,A., Oudet,P.and Sentenac,A. (1989) EMBO J. 8, 3815-3824.
20 Huibregtse,J.M., Evans,C.F. and Engelke,D.R. (1987) Mol. Cell. Biol. 7, 3212-3220.
21 Griffith,J., Hoschchild,A. and Ptashne,M. (1986) Nature 322, 750-753.
22 Wu,H.M. and Crothers,D.M. (1984) Nature 308, 509-513.
23 Robertson,C.A. and Nash,H.A. (1988) J. Biol. Chem. 263, 3554-3557.
24 Shaw,K.J. and Olson,M.V. (1984) Mol. Cell. Biol. 4, 657-665.
25 Saiki,R., Scharf,S., Faloona,F., Mullis,K., Horn,G., Erlich,H. and Arnheim,N. (1985) Science 230, 1350-1354.
26 Sanger,F. and Coulson,A.R. (1978) FEBS Lett. 87, 107-110.
27 Newman,A.J., Ogden,R.C. and Abelson,J. (1983) Cell 35, 117-125.
28 Strobel,M.C. and Abelson,J. (1986) Mol. Cell. Biol. 6, 2663-2673.

29 Huibregtse,J.M and Engelke,D.R. (1986) Gene 44, 151-158.
30 Piper,P.W. and Straby,K.B. (1989) FEBS Lett. 250, 311-316.
31 Evans,C.F. and Engelke,D.R. (1990) Meth. Enzym. 181, 439-450.
32 Evans, C.F. (1990) Ph.D. thesis, The University of Michigan
33 Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor University Press, Cold Spring Harbor.
34 Tabor,S., Huber,H.E. and Richardson,C.C. (1987) J. Biol. Chem. 262, 16212-16223.
35 Borowiec,J.A. Zhang,L., Sasse-Dwight,S. and Gralla,J.D. (1987) J. Mol. Biol. 196, 101-111.
36 Hayatsu,H. and Ukita,T. (1967) Biochem. Biophys. Res. Comm. 29, 556-561.
37 Borowiec,J.A. and Hurwitz,J. (1988) EMBO J. 7, 3149-3158.
38 Marzouki, N., Camier, S., Ruet, A., Moenne, A. and Sentenac, A. (1986) Nature 323:176-178
39 Pieler,T., Hamm,J. and Roeder,R.G. (1987) Cell 48, 91-100.
40 O'Halloran,T.V., Frantz,B., Shin,M.K., Ralston,D.M. and Wright,J.G. (1989) Cell 56, 119-129.
41 Camier,S., Baker,R.E., and Sentenac, A. (1990) Nucleic Acids Res. 18, 4571-4578.
42 Kassavetis,G.A., Braun,B.R., Nguyen,L.H. and Geiduschek,E.P. (1990) Cell 60, 235-245.