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Interaction of tRNA transcription factors with satellite I DNA from *Xenopus laevis*

(Recombinant DNA; DNA binding proteins; yeast RNA polymerase III; repetitive class III promoters; footprinting)

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SUMMARY

A cloned repeat of *Xenopus laevis* satellite I DNA was tested for the ability to form stable complexes with tRNA transcription factors in vitro. In template exclusion studies, the satellite I DNA competed efficiently with a tRNA gene for binding of yeast RNA polymerase III transcription factors. DNase I footprinting further showed that transcription factor TF IIIC alone bound to satellite I DNA at both the A block and B block consensus promoter sequences immediately downstream from the transcription start point. The strength and position of these associations indicate that satellite I DNA is a potential site for association of the same DNA-binding proteins that activate tRNA gene transcription.

INTRODUCTION

Satellite I DNA from *Xenopus laevis* is a highly reiterated element of 741 bp that exists as dispersed tandem clusters (Lam and Carroll, 1983). Sequence analysis of independently isolated repeats (Lam and Carroll, 1983; Ackermann, 1983) revealed the presence of appropriately spaced DNA segments homologous to the conserved 5′ and 3′ internal promoter elements (A and B blocks) found in eukaryotic tRNA genes (Traboni et al., 1982; Sharp et al., 1981) and other highly repetitive eukaryotic sequence elements (Duncan et al., 1979; Elder et al., 1981; Fuhrman et al., 1981; Fritsch et al., 1981; Perez-Stable et al., 1984, Sutcliffe et al., 1984). Transcription of the cloned satellite DNA by cellular extracts and by injection into *Xenopus* oocytes (Ackerman, 1983; Wakefield et al., 1983; Andrews et al., 1984) has confirmed that these promoter elements do, in fact, compete for the binding of RNA polymerase III transcription factors and direct transcription initiation immediately upstream from the A

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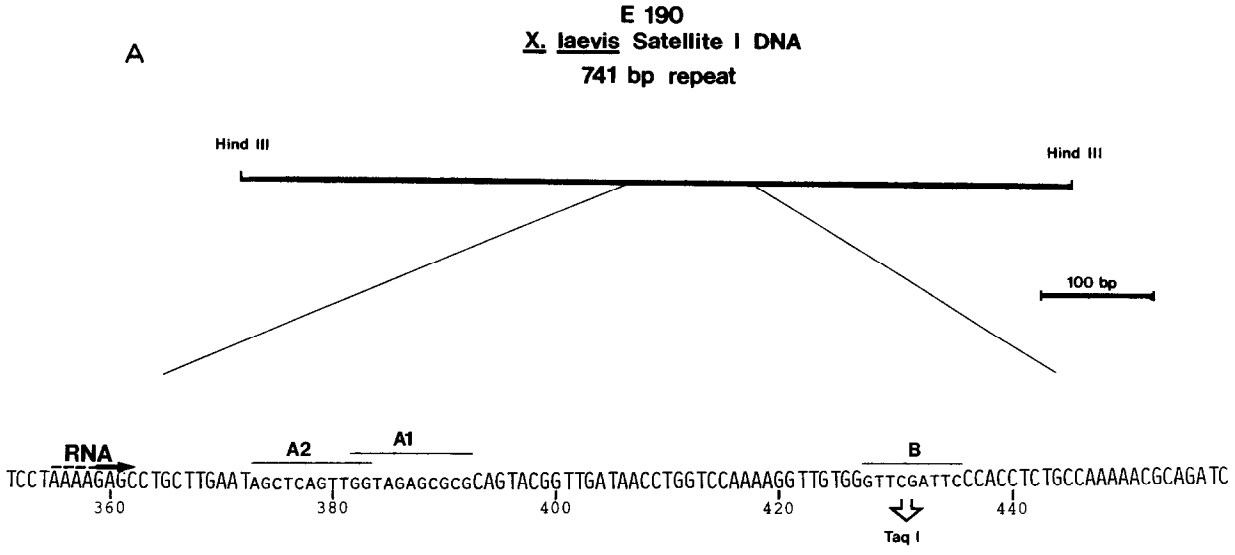
Abbreviations: bp, base pair(s); Δ, deletion; nt, nucleotide(s); PA, polyacrylamide; SDS, sodium dodecyl sulfate; TF, transcription factor.



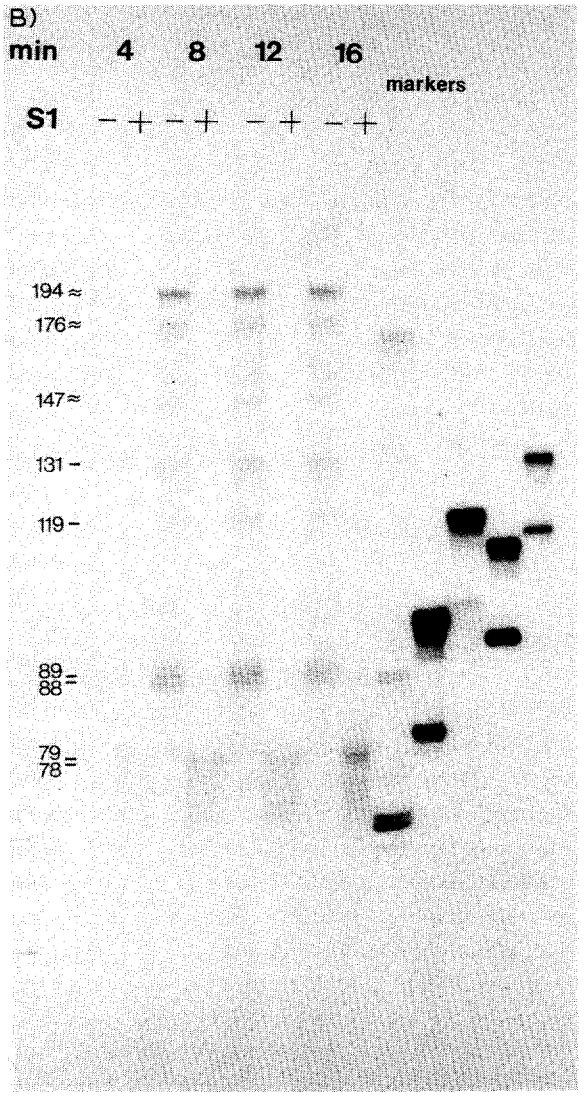


Fig. 1. Time course of pE190 transcription by the nuclear extract and S1 nuclease analysis of RNA. (A) The structure and sequence of plasmid pE190 containing a satellite I DNA repeat in the *Hin*dIII site of pBR322 has been published previously (Lam and Carroll, 1983). The nucleotide sequence of the nontemplate strand is shown in the region containing the major transcription start points and the RNA polymerase III promoter consensus sequences (small caps). In addition to the A block promoter consensus (PuPuPyNNAPuPyGG) noted earlier (region A2, Wakefield et al., 1983; Andrews et al., 1983) a second region of lesser homology is indicated (A1). A probe for S1 nuclease analysis of the RNA transcripts from this region was prepared by cloning the fragment extending from the left-hand *Hin*dIII site to the indicated *Taq*I site between the *Hin*dIII and *Acc*I sites of M13mp18 virus. Viral DNA contained a single DNA strand complementary to the sequence shown. (B) Plasmid pE190 was transcribed by the *S. cerevisiae* extract in the presence of [α-^{32}P]UTP for 3 min, after which a 50-fold excess of unlabeled UTP was added to quench the incorporation of label, and the incubations continued an additional 8 min. Aliquots were taken for analysis at 4, 8, 12 and 16 min after the addition of extracts. The low level of transcripts seen after 4 min is typical of the 1.5–3-min lag in transcription initiation (Engelke et al., 1985). The subsequent transcription initiation rate was estimated to be greater than 0.6 transcripts per gene per min by determining Cherenkow radiation in the product bands. Radiolabeled E190 RNA aliquots were either analyzed directely by size ('minus' S1 lanes) or first pre-incubated with 10 μg unlabeled, single stranded, viral S1 probe (panel A) and treated with S1 nuclease ('plus' S1 lanes) prior to electrophoretic analysis. The surviving RNA showed a major start point 78 nt upstream from the *Taq*I site and other products up to 8 nt shorter. The positions of these initiation sites are shown relative to the A and B block sequences in panel A. The sizes of the RNA transcripts, shown in nt to the left of the panel, were determined by using as standards the precursor and end-matured forms of (left to right in 'markers' lanes): $tRNA^{Arg/Asp}$ (71 to 170 nt; Engelke et al, 1985) and four pairs of $tRNA^{Leu}$ with various lengths deleted from the inter-

block consensus. Here the association between partially purified RNA polymerase III transcription factor TFIIIC (Engelke et al., 1985; Huibregtse et al., 1987) and the promoter elements is shown to be characteristic of a tRNA-like transcription unit.

EXPERIMENTAL AND DISCUSSION

(a) Satellite I DNA transcription in vitro

The monomer clone of satellite I DNA used in these experiments, pE190, has been described previously (Lam and Carroll, 1983; gift of Dana Carroll, University of Utah). The structure of the 741-bp repeat is shown in Fig. 1A along with the nucleotide sequence in the region of the A block and B block RNA polymerase III promoters. In addition to the A2 consensus sequence noted previously (Ackerman, 1983; Andrews et al., 1984) a second overlapping region of lesser homology to the A consensus is indicated (A1). The ability of these sequences to efficiently bind the tRNA promoter recognition factor, TFIIIC, could be tested using transcription factor fractions isolated from yeast nuclei (Huibregtse et al., 1987) because recognition of the tightly conserved tRNA gene internal promoters by transcription components from evolutionarily distant species is known to occur efficiently (Sprague et al., 1980; Dingermann et al., 1982). Although the physiological significance of satellite I DNA transcription by heterologous or even homologous extracts is uncertain (see section **d**), transcription of the E190 sequences was characterized to ensure that factor-DNA complexes potentiated transcription initiation upstream from the promoter sites in a manner similar to that observed using homologous components.

Transcription of the satellite I repeat routinely produced multiple RNA species of 87 to approx. 194 nt, consistent with the multiple transcripts observed previously with *Xenopus* extracts (Andrews et al., 1984). To map the transcription start point(s) and to test for the possibility that the various bands arise from nucleolytic RNA processing, a pulse-chase time course of transcription was performed (Fig. 1B). One-half of the RNA products from each time point were analyzed directly by size (−S1 lanes) and showed no obvious precursor-product relationship among the bands. Since tRNA precursors from both yeast and *Xenopus* are subject to removal of their 5′ and 3′ termini and to splicing in this extract (Newman et al., 1983; Engelke et al., 1985; Strobel and Abelson, 1986), this observation suggests that the E190 transcripts do not contain tertiary structure appropriate for recognition by pre-tRNA processing enzymes.

To identify the transcription start point(s) the other half of the radiolabeled pE190 transcripts from each time point were annealed to single-stranded M13 viral DNA containing as an insert the template strand between the leftward *Hin*dIII site and the *Taq*I site within the B block. Digestion with S1 nuclease (+S1 lanes) gave protected RNA fragments consistent with transcription initiation within a run of five A residues upstream of the A blocks (indicated in Fig. 1A). The heterogeneity observed in the 5′ terminus probably represents true heterogeneity in initiation since the only major runoff transcripts when the pE190 template is truncated downstream of the B block also correspond to diffuse initiations in this region (D.R.E., unpublished observation). The longest of these transcripts initiates 4 bp upstream from the transcription start point observed in *Xenopus*, but this difference was not unexpected given that heterologous transcription extracts fre-

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vening sequence (99 to 81, 118 to 100, 112 to 94, 131 to 133 nt; Strobel and Abelson, 1986). Uncertainty in the exact assignment of pE190 transcript sizes longer than 131 nt was due to the relatively small number of longer markers. **Methods**: Plasmid templates pE190 and pUCtRNA^{Leu}-ΔIVS and preparation of *S. cerevisiae* transcription extracts have been described (Lam and Carroll, 1983; Strobel and Abelson, 1986; Huibregtse et al., 1987). Transcription time-course reactions were assembled in 100 μl containing 50 mM HEPES (*N*-2-hydroxyethylpiperizine-*N*′-2-ethanesulfonic acid, pH 7.9), 120 mM KCl, 8 mM $MgCl_2$, 0.2 mM dithiothreitol, 1 mM phosphoenolpyruvate, 200 μM each ATP, GTP and CTP, 20 μM UTP including 10 μCi [α-^{32}P]UTP (New England Nuclear), 2.5–4 μg plasmid template and 20 μl yeast extract. 20 μl aliquots were withdrawn at the indicated times, stopped and digested for 90 min at 50°C with the addition of 4 μl stop mix [1 mg/ml proteinase K (Beckman), 2% SDS, 100 mM EDTA, pH 8.0] and precipitated with ethanol. Ten μg of viral S1 probe DNA was annealed to one-half of the RNA from each time point and digested for 30 min at 18°C with 10 units of S1 nuclease. Reactions were halted with stop mix, precipitated with ethanol and analyzed by size on denaturing 10% PA gels (Engelke et al., 1985).

quently do not recognize upstream promoter sequences (Sprague et al., 1980; Dingermann et al., 1982). The longest transcript (approx. 194 nt) corresponds to transcription termination at the first RNA polymerase III terminator (five T residues) 108 nt downstream from the B block. The reason for the apparent transcription pauses or terminations giving rise to the shorter trancripts was not obvious from inspection of the nucleotide sequence.

(b) Competion between satellite I DNA and tRNA genes for transcription factors

Template exclusion assays (Fuhrman et al., 1984; Baker and Hall, 1984) were performed to affirm the ability of the satellite I DNA to stably bind tRNA gene transcription components and the requirement for such binding in transcription. A tRNA gene with a strong in vitro promoter, $tRNA^{Leu}$ (SUP53) with a deleted intervening sequence (Strobel and Abelson, 1986), was added to the extract either in increasing amounts before the addition of pE190 (Fig. 2, left-hand lanes) or after increasing concentrations of pE190 had been preincubated with the extract (right-hand lanes). DNA concentration was held constant by the inclusion of plasmid vector pUC9 in the reactions. In both cases transcription of the second template by 4 μl of extract was excluded more than 50% by 60 fmol of the first template and entirely excluded by 260 fmol. Thus, even though transcription initiation on the E190 DNA by RNA polymerase III was routinely only 10% to 20% as frequent as transcription of the tRNA gene, both templates efficiently bound one or more required transcription factors into stable complexes. The reason for the difference in initation frequency between the two templates is not clear, but may be related to the lack of appropriate yeast transcription signals upstream from the E190 internal promoter region (Johnson and Raymond, 1984).

(c) Transcription factor IIIC binds to the bipartite internal promoter

To identify the sites of transcription attachment, partially purified transcription factors TFIIIB and TFIIIC (Engelke et al., 1980; Segall et al., 1980; Fuhrman et al., 1984) containing no detectable cross-contamination (Huibregtse et al., 1987) were used in DNase I footprinting experiments with either satellite I DNA or the $tRNA^{Leu}$-ΔIVS gene (Fig. 3A). As observed previously for the $tRNA^{Leu}$ gene (Klemenz et al., 1982; Stillman et al., 1985; Huibregtse et al., 1987), TFIIIC afforded DNase I protection at the A and B blocks of both DNAs in the presence or absence of TFIIIB. The extent of this protection relative to the internal promoter sequences is depicted in Fig. 3B. The A2 sequence is likely to be the one primarily used for binding the transcription factor(s) since the upstream DNase I protection is centered over this region, similar to the case for the tRNA gene footprints (Stillman et al., 1984; Huibregtse et al., 1987). This conclusion is reinforced by the fact that the E190 transcription start points are approximately the same distance upstream from the A2 consensus as the tRNA start points are from the tRNA A block. We cannot, however, rule out the possibility that the A1 sequence plays some role in the efficiency of factor binding or transcription initiation. Evidence for protection being due to the identical factor(s) binding to both DNAs was provided by preincubation of the factors with an unlabeled excess (260 fmol) of pE190 DNA competitor in tRNA gene footprinting reactions or of $ptRNA^{Leu}$-ΔIVS DNA competitor in E190 footprinting reactions (Fig. 3A, '+' competitor DNA lanes).

(d) Significance

The functions of *Xenopus* satellite I DNA in vivo are obscure. Examination of endogenous RNA from oocytes, embryos and somatic cells has shown, at best, low levels of satellite I transcripts (Wakefield et al., 1983). The lack of highly reiterated satellite I DNA in *X. borealis* and *X. mulleri* (Lam and Carroll, 1983) also argues that the majority of the repeats are entirely dispensable. One possible explanation for these observations is that this RNA is expressed only in a tissue or during a developmental stage not yet examined and that only a small number of repeats need be activated. Alternatively, several explanations for the presence of reiterated RNA polymerase III promoter elements have been suggested that assume recognition by site-specific DNA binding proteins, but that do not necessarily require the production of stable RNA products (Jelinek and Leinwand, 1978; Van Arsdell et al., 1981; Jagadees-

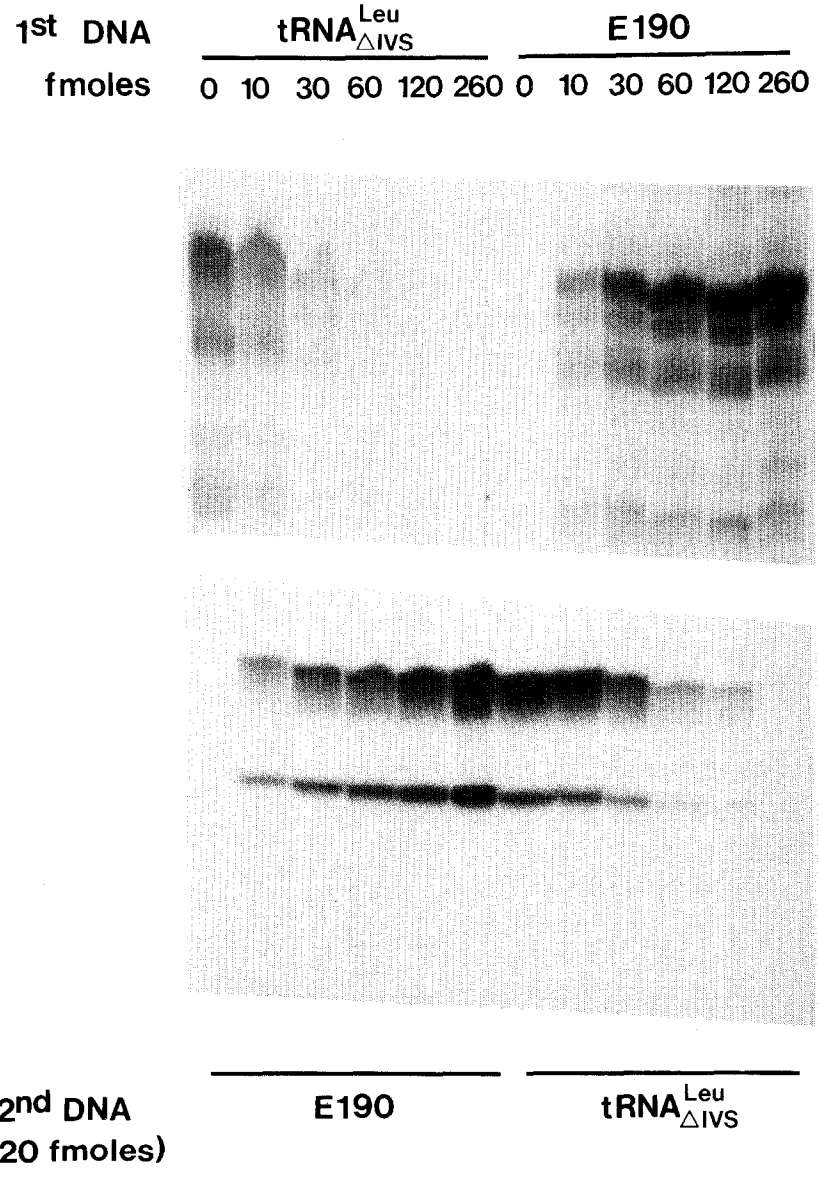
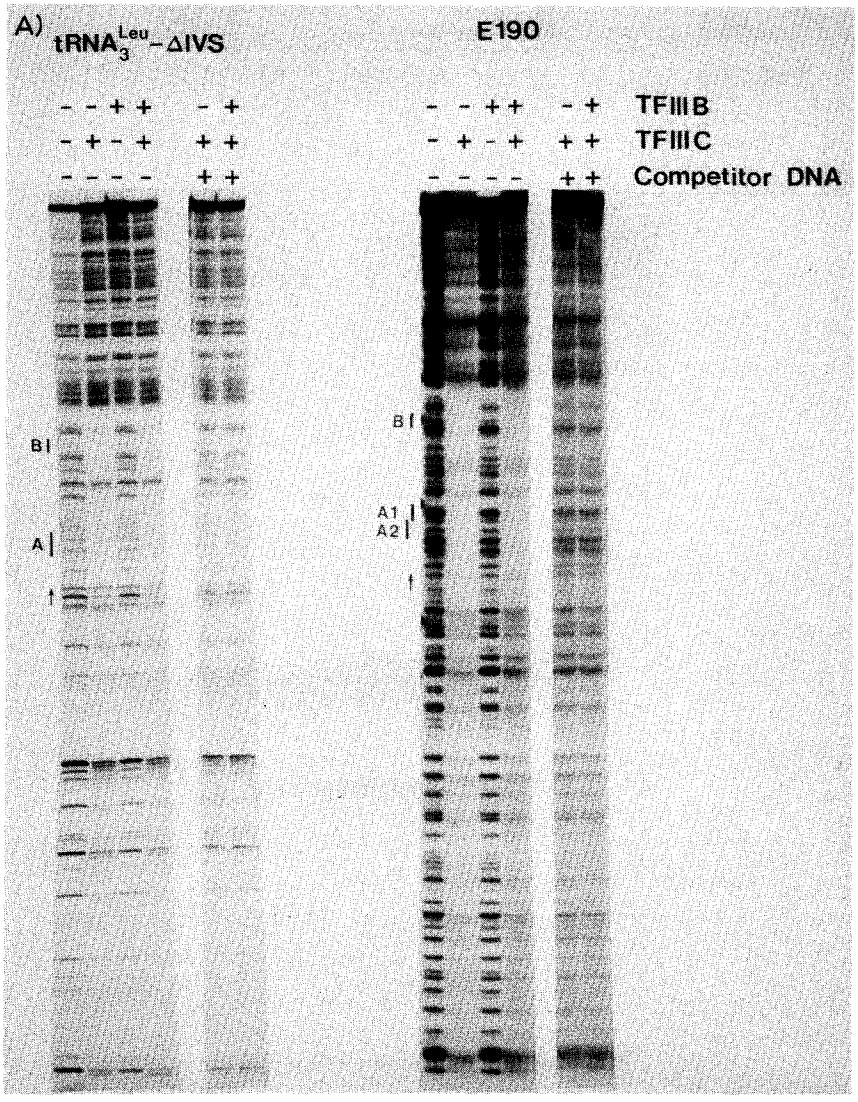


Fig. 2. Mutual template exclusion by E190 and $tRNA^{Leu}$ DNA. Transcription reactions were performed as described in Fig. 1 except that individual 20-μl reactions were assembled with the first template indicated in the absence of [α-^{32}P]UTP and incubated at 30°C for 3 min. Plasmid pUC9 was added to the reactions to equalize the DNA concentration at 30 μg/ml. After the preincubation, 220 fmol of the second plasmid template and [α-^{32}P]UTP were added. Incubations continued for 15 min before the reactions were stopped and RNA analyzed by electrophoresis through denaturing 10% PA gels (Sanger and Coulson, 1978). The data shown were derived from two radioautography exposures of the same gel for 12 h (lower panel) or 40 h (upper panel) to adjust for differences in transcription efficiency. The $tRNA^{Leu}$-ΔIVS gene was chosen for this experiment because both the primary transcript (upper band, lower panel) and the end-matured processing product (lower band, lower panel) migrated faster than all of the pE190 transcripts (upper panel).

waran et al., 1981; Sutcliffe et al., 1984; Andrews et al., 1984). The results presented here show that the RNA polymerase III transcription factor TFIIIC binds tightly to the satellite I A block and B block promoter sequences without mediation by *Xenopus* satellite I-specific components. This suggests that whatever function the satellite I sequences play may be mediated by the same class of factor that forms stable complexes with the tRNA genes.



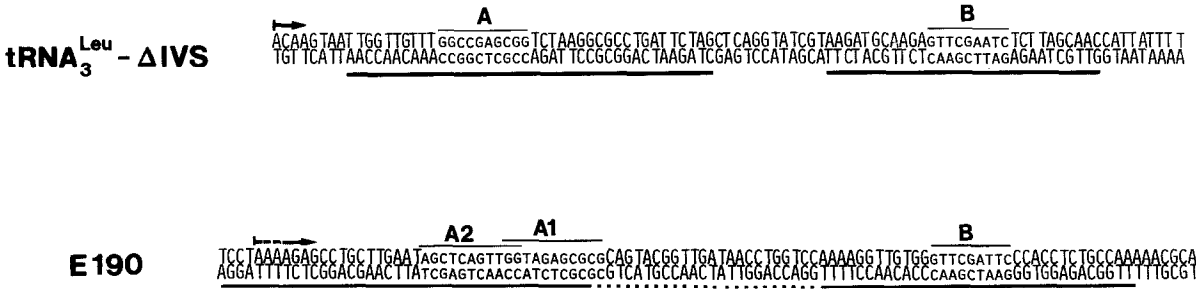


Fig. 3. DNase I protection of A and B block promoter sequences by transcription factors TFIIIB and TFIIIC. (A) DNase I footprinting (Galas and Schmitz, 1978) was performed using tRNA gene transcription factors TFIIIB and TFIIIC. Where the inclusion of competitor DNA is indicated, the pUC9 carrier was replaced with either 260 fmol of plasmid pE190 in the tRNA gene footprinting or 260 fmol of plasmid pUCtRNA^{Leu}-ΔIVS in the E190 DNA footprinting. For both the tRNA^{Leu}-ΔIVS gene and the E190 DNA the positions of the A and B blocks are indicated to the left of the panels. Arrows to the left of each panel indicate the position and direction of transcription initiation. Protection of the tRNA gene promoters is essentially in agreement with previous reports (Klemenz et al., 1982; Stillman et al., 1985; Huibregtse et al., 1987). The reason for minor differences in the DNase I digestion profile outside the tRNA gene in the absence

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or presence of the TFIIIC fraction is unknown. The differences were not removed by the inclusion of specific competitor DNA and similar effects have been seen with DNA fragments not harboring RNA polymerase III promoters (not shown). **(B)** Minimum regions of satellite I DNA and tRNA gene protected by the presence of TFIIIC are indicated by solid lines below the sequence. In regions where no cleavages exist in the naked DNA controls, positions between protected cleavages have been assigned as protected. Positions between a protected and non-protected cleavage have been assigned as unprotected. E190 DNA between the A and B regions was reproducibly subject to reduced DNase I cleavage (dotted line), but not to full protection. Major transcription start points are indicated by arrows. **Methods**: Fractionation of yeast tRNA gene transcription factors TFIIIB and TFIIIC is described elsewhere (Huibregtse et al., 1987). Linear DNA fragments containing satellite I (E109) DNA or the $tRNA^{Leu}$-ΔIVS gene were end-labeled by filling in restriction site overhangs with [α-^{32}P]dATP (New England Nuclear) using the Klenow fragment of *E. coli* DNA polymerase I (Bethesda Research Laboratories). E190 fragment from a *Sma*I-*Hin*dIII subclone of E190 (inserted between the *Sma*I and *Hin*dIII sites of the pUC9 polylinker) was labeled 225 bp uspstream from the A2 sequence at the vector *Eco*RI site. The tRNA gene fragment was labeled 240 bp upstream from the A block promoter at the vector *Eco*RI site. Footprinting reactions were performed as described by Newman et al. (1983) with minor modifications. Binding reactions were carried out at 22°C for 10 min using 10 fmol of labeled DNA fragment, 0.8 μg of plasmid pUC9 carrier or plasmid competitor and enough of the indicated transcription factor (TFIIIB or TFIIIC) to saturate an in vitro transcription reaction containing 125 fmol of tRNA gene. DNase I (Worthington) was added to a final concentration of 1.0 μg/ml for 30 s at 22°C. The reactions were halted with stop mix and the DNA was ethanol-precipitated and subjected to electrophoresis through denaturing 10% polyacrylamide gels (Sanger and Coulson, 1978). Restriction endonuclease cleavages and nucleotide sequencing reactions using the end-labeled DNAs were used as markers to determine the positions of DNase I cleavages.

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