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Differential binding of a *S. cerevisiae* RNA polymerase III transcription factor to two promoter segments of a tRNA gene

David J. Stillman* and E. Peter Geiduschek

Department of Biology, University of California, San Diego, La Jolla, CA 92093, USA

*To whom reprint requests should be sent
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A *Saccharomyces cerevisiae* protein fraction which binds specifically to the internal promoter regions of genes that are transcribed by RNA polymerase III is shown to function as a transcription factor. We postulate that the stable DNA binding of the factor confers stability on polymerase III transcription complexes. Analysis of the binding by DNase 'footprinting' distinguishes three segments of the *S. cerevisiae* $tRNA_3^{Leu}$ gene: a region surrounding the so-called A block of the internal promoter, a region surrounding the B block and an intermediate segment. Binding to the A and B block regions is connected, but the B block region exerts a dominant effect.

Key words: RNA polymerase III/bipartite promoter/tRNA genes/DNA-binding proteins/footprinting

Introduction

Initiation of transcription by eucaryotic RNA polymerases requires the participation of multiple accessory factors. The work reported here deals with genes transcribed by RNA polymerase III; the characteristically short polymerase III transcription units have promoters that are transcribed. Tests of transcription activity of tRNA genes, which have been altered *in vitro* by deletions, insertions or nucleotide substitutions, in cell-free transcription systems or *in vivo* (Carrara *et al.*, 1981; Galli *et al.*, 1981; Hofstetter *et al.*, 1981; Sharp *et al.*, 1981; Ciliberto *et al.*, 1982a, 1982b; Koski *et al.*, 1980, 1982; Mattoccia *et al.*, 1983) have defined two distinct, well-separated elements of tRNA gene promoters, each ~10 bp long, referred to as the 'A' and 'B' blocks (for review, see Hall *et al.*, 1982; Ciliberto *et al.*, 1983). A 5 bp long, conserved sequence near the B block also contributes quantitatively to promoter strength (Allison *et al.*, 1983). Sequences upstream of transcriptional start sites can also modulate promoter activity (Sprague *et al.* 1980; De Franco *et al.*, 1981; Dingermann *et al.*, 1982; Shaw and Olson, 1983; Hipskind and Clarkson, 1983). The distances between the A and B blocks are not constant in natural tRNA genes and can be varied or enlarged considerably without abolishing promoter activity (Johnson *et al.*, 1980; Ciliberto *et al.*, 1982b; Carrara *et al.*, 1981; Galli *et al.*, 1981). Moreover, recent work (Folk and Hofstetter, 1983) suggests that the dyad symmetry in the anticodon stem of the tRNA gene, and hence the potential for cruciform structure in DNA, may be a significant determinant in promoter strength of a *Xenopus laevis* $tRNA^{Met}$ gene. Clearly, the polymerase III promoters are larger and more diverse than *Escherichia coli* promoters whose mutations are confined to the 6 bp '−35' and '−10' consensus sequences or their immediate neighbors (Youderian *et al.*, 1982), well within the DNA-binding domain of RNA polymerase itself. The dispersed nature of tRNA gene promoter mutations must reflect some spatial property of protein and DNA in promoter complexes: unless those complexes are extraordinarily long, the DNA must be bent or folded.

We have studied the interaction of certain yeast proteins with genes that are transcribed by RNA polymerase III (Klemenz *et al.*, 1982). Because of the locations and characteristics of the DNA-binding sites, we have assumed that the DNA-binding proteins would turn out to be involved in tRNA gene transcription. Evidence supporting the validity of that assumption is now at hand. Here, we analyze the interaction of the DNA-binding proteins with a tRNA gene in more detail. We also demonstrate that the fraction, which contains these proteins, functions as a polymerase III transcription factor.

Results

Footprints

First, we examined the DNase I footprint generated in a *Saccharomyces cerevisiae* $tRNA_3^{Leu}$ gene by the proteins in the previously described yeast fraction (which we here call PC 200). The posterior gene segments surrounding the 'B blocks' of several genes are strongly and stably protected from DNase I by these proteins (Klemenz *et al.*, 1982). However, protection from DNase I is clearly not confined to that part of these genes (Klemenz *et al.*, 1982, Figure 2). Figure 1A, lanes 3 and 4 show a footprint of a 341-bp *RsaI/MspI* fragment containing the $tRNA_3^{Leu}$ gene. The following are the salient features of the DNase I protection pattern (Figure 1B). (i) A protected region of DNA located near the 3′ end of the gene, designated '*b*', is bracketed by unprotected or enhanced cleavage sites at base pairs 82 and 125. Between base pairs 88 and 109, the DNA is uniformly and completely protected; at base pair 113, the DNA is very strongly (but not completely) protected. The *b* region surrounds the B block which extends from base pair 93 to 103. (ii) A second segment protected from DNase I digestion located at the 5′ end of the gene, termed the '*a* region', is flanked on one side by an enhanced site at base pair 36 and on the other by somewhat enhanced cleavage at base pair −9. Between −1 and +31, the DNA is approximately uniformly, but not completely, protected. The *a* region surrounds the A block, which extends from base pair 8 to 18. (iii) A DNA segment with a complex footprint pattern is bounded by the enhanced cleavage sites at base pair 36 and unprotected base pair 82. This segment of the $tRNA_3^{Leu}$ gene contains the 31-bp intervening sequence and the contiguous anticodon loop. We refer to it as the *i* (intermediate) region. The protection pattern in this region is highly reproducible in repeated experiments with the footprint-conferring fraction PC 200 (see Figures 3 and 6 below, for example). There are clearly enhanced cleavages at base pairs

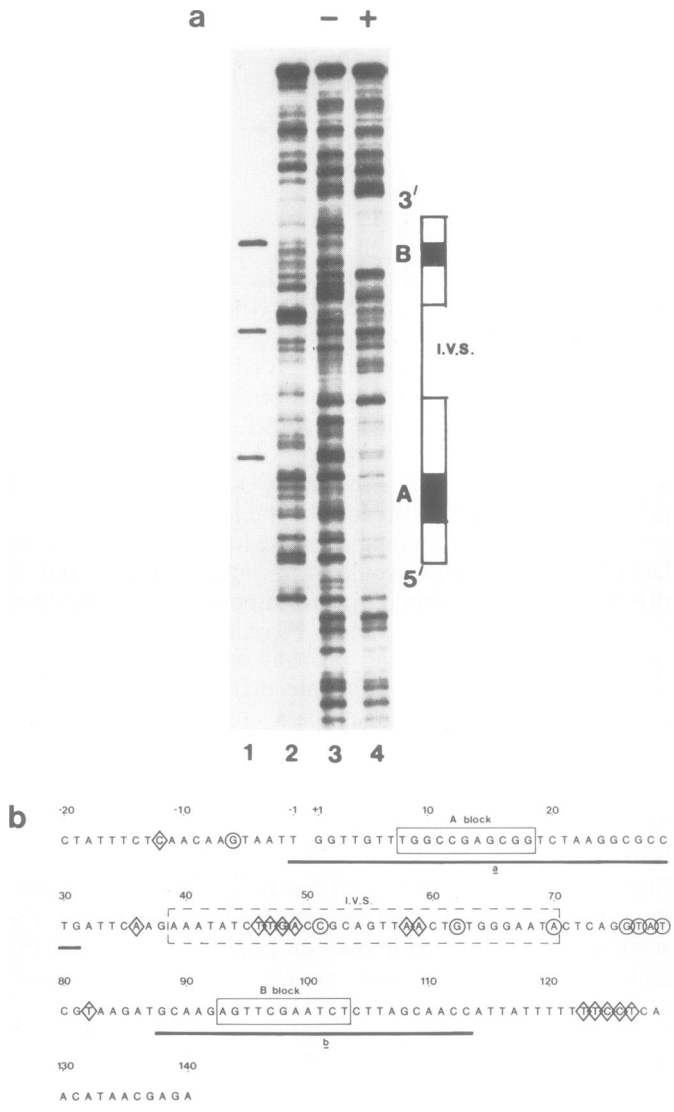

Fig. 1. The footprint of the *S. cerevisiae* $tRNA_3^{Leu}$ gene. **(A)** The 341-bp *RsaI/MspI* fragment from YEp13 was 5′ end-labeled (in the non-transcribed strand) at the *RsaI* site, 73 bp from the base pair corresponding to the 5′ end of the mature tRNA. **Lane 1** contains a mixture of *TaqI*, *HpaI* and *DdeI*-digested probes. **Lane 2** contains the G-sequencing ladder of the probe (Maxam and Gilbert, 1980). The probe was incubated in the absence **(lane 3)** or presence **(lane 4)** of 1 μl of protein fraction PC 200, and digested with DNase I as described in Materials and methods. The diagram at the side shows the A and B blocks (filled rectangles) contained within the coding region for the mature tRNA (open rectangles); the single line represents the intervening sequence. **(B)** The sequence of the gene (Andreadis *et al.*, 1982) with the A and B blocks and intervening sequence marked. The *a* and *b* regions of the footprint are also noted. Diamonds enclose sites corresponding to enhanced bands, and circles enclose protected bands at the boundaries of *a* and *b* and within *i*. the non-transcribed strand is shown. (+1) designates the 5′ end of mature tRNA.

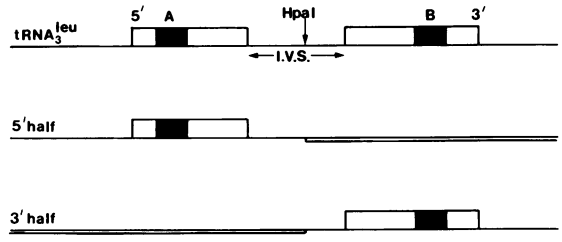

Fig. 2. The $tRNA_3^{Leu}$ gene, its 5′ half and 3′ half subclones. pJD137 (Johnson *et al.*, 1980) is a 2.5-kbp insert containing a $tRNA_3^{Leu}$ gene into the *Eco*RI site of pBR322. The 5′ and 3′ half subclones were inserted by Raymond and Johnson (1983) into the *Hind*III site of pBR322, converting the *Hpa*I site into an *Hind*III site through the use of an oligonucleotide linker. The 450-bp 5′ half subclone is oriented in the vector such that a transcript initiating at the correct 5′ end would continue towards the *Bam*HI site. The 2-kbp 3′ half subclone is in the opposite orientation. Rectangles represent segments coding for mature tRNA, filled rectangles the A and B blocks, and closely spaced double lines pBR322 sequences. The intervening sequence (I.V.S.) is also indicated.

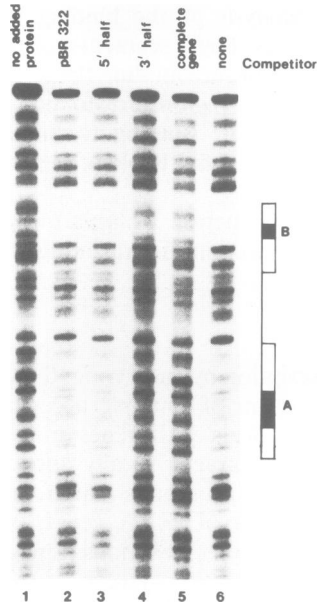

Fig. 3. Footprint competition. The complete $tRNA_3^{Leu}$ gene probe is a 341-bp *RsaI/MspI* fragment from YEp13. The sample displayed in **lane 1** contained no added yeast protein; **lanes 2–6** contained 1 μl of fraction PC 200. **Lanes 2–5** contained 40 fmol of plasmid competitor DNA: pBR322 **(lane 2)**; pJD 5′ half gene **(lane 3)**; pJD 3′ half gene **(lane 4)**; complete $tRNA_3^{Leu}$ gene, pJD137 **(lane 5)**. Since the plasmids used as competitors in **lanes 2–5** have different sizes, pBR322 was added to bring the total weight of added DNA to 200 ng. **Lanes 1** and **6** had only 50 ng pBR322 as carrier. The diagram at the side is described in the legend to Figure 1.

46–49, there is strong protection at base pair 51, an unprotected cleavage site at base pair 58 or 59, strong protection at base pair 62, less strong protection at base pair 70 and strong protection at base pairs 76–79. Given the complexity of the pattern, one might conjecture (see Discussion) about the spatial arrangement of this segment of the gene relative to the binding protein(s). However the PC 200 fraction contains non-specific DNA-binding proteins and the competition between non-specific and specific DNA-binding proteins can affect protection patterns (Fuhrman *et al.*, 1984). As a precaution, unlabeled carrier DNA (pBR322) has been added to all the footprint reactions shown here (see Materials and methods). When that carrier DNA is omitted, the footprint pattern in the *i* region changes significantly (cf. Figure 4, Klemenz *et al.*, 1982).

A comparison between the footprints generated in a *S. cerevisiae* $tRNA^{Gln}$ gene and the $tRNA_3^{Leu}$ gene is instructive. The $tRNA^{Gln}$ gene lacks the large intervening sequence and large variable loop of the Leu_3 gene; thus, the spacing of the so-called A and B blocks differs in these genes. When the $tRNA^{Gln}$ gene was used as the probe, the entire gene was protected (Klemenz *et al.*, 1982, Figure 2C); the tripartite pattern of the $tRNA_3^{Leu}$ gene (Figure 1) was not seen. The two footprints could be matched to each other in the following way.

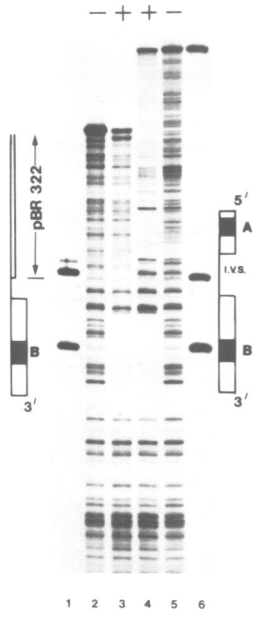

Fig. 4. Comparison of the footprints of the 3′ half and complete $tRNA_3^{Leu}$ genes. The 3′ half probe **(lanes 1–3)** is a 405-bp *Xba*I/*Eco*RV fragment from pJD 3′ half, 5′ end-labeled at the *Xba*I site. The complete gene probe **(lanes 4–6)** is a 725-bp *Xba*I/*Eco*RI fragment from pJD 137, 5′ end-labeled at the *Xba*I site. Both probes are labeled in the transcribed strand; the probes for Figures 1, 3 and 6 are labeled in the non-transcribed strand. The probes were incubated in the absence **(lanes 2, 5)** or presence **(lanes 3, 4)** of the PC 200 fraction before footprinting. **Lane 1** contains a mixture of separate digests of the 3′ half probe with *Hind*III and *Taq*I. **Lane 6** contains a mixture of separate digests of the complete gene probe with *Hpa*I and *Taq*I. The schematic diagrams show the positions of the A and B blocks (filled rectangles) within the coding sequence (open rectangle), the intervening sequence (single line), and the pBR322 sequence in the 3′ half clone (closely spaced double lines). Positions within the probes were determined using restriction endonuclease-cleaved probes **(lanes 1, 6)**, *Hinf*I pBR322 markers (data not shown), and sequencing ladders (Maxam and Gilbert, 1980, data not shown).

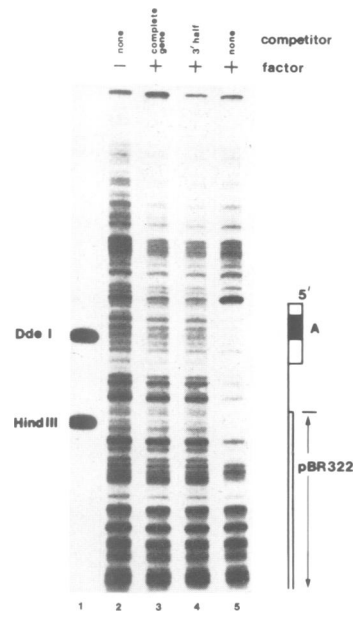

Fig. 5. Footprint of the $tRNA_3^{Leu}$ 5′ half gene. The probe is a 640-bp *Eco*RV/*Eco*RI fragment from pJD 5′ half, 5′ end-labeled at the *Eco*RV site. The probe was incubated in the absence **(lane 2)** or presence **(lanes 3–5)** of the PC 200 fraction. 80 fmol of pJD137 **(lane 3)** or pJD3′ half **(lane 4)** plasmid DNA was added as competitor. **Lane 1** shows a mixture of separate digests of the probe with *Dde*I and *Hind*III. The diagram at the side shows the position of the A block (filled rectangle) within the remaining coding sequence (open rectangle), the intervening sequence (single line), and the pBR322 sequences (closely spaced double lines). Positions within the probe were determined using restriction endonuclease-cleaved probe **(lane 1)** *Hinf*I pBR322 markers (data not shown), and sequencing ladders (Maxam and Gilbert, 1980, data not shown).

(i) The complete protection of the $tRNA^{Gln}$ gene between base pairs 38 and 74 matched the *b* region protection pattern of the $tRNA_3^{Leu}$ gene, as already noted (Klemenz *et al.*, 1982). (ii) The $tRNA^{Gln}$ gene was strongly but not absolutely protected between base pairs −6 and 38; enhanced cleavage was seen at −9. This segment of the $tRNA^{Gln}$ gene footprint resembled the *a* region pattern of the Leu_3 tRNA gene. (iii) The protection and enhancement-alternating *i* segment of the $tRNA_3^{Leu}$ footprint could not be seen in the $tRNA^{Gln}$ gene.

We used subclones containing only half of the $tRNA_3^{Leu}$ gene (Figure 2) to characterize further the relationship between the *a* and *b* regions of the gene and their binding proteins. Each half gene contains a symmetric sequence, contributed by a *Hind*III linker, at the pBR322 junction. The clones were kindly made available by J.Johnson (Raymond and Johnson, 1983). The ability of the half gene subclones to compete for the specific proteins responsible for the $tRNA_3^{Leu}$ gene footprint is tested in Figure 3. A footprint in the absence of added competitor DNA is shown in lane 6. Added pBR322 (lane 2) or 5′ half gene (lane 3) did not compete, while the same molar concentration of the 3′ half (lane 4) and the complete gene (lane 5) competed equally effectively. To measure the binding of different DNAs for the footprint factor, we titrated the ability of unlabeled competitor DNA to block a footprint; the 3′ half competed as efficiently as the complete gene (data not shown). Note that the 3′ half subclone competed for the entire footprint (Figure 3), even though the competitor lacks the *a* region sequences and part of the *i* region.

An end-labeled DNA probe was prepared from the 3′ half subclone and used in a footprint experiment (Figure 4). The B block protection on the 3′ half probe (lanes 2, 3) and the complete gene probe (lanes 4, 5) appeared to be identical, but the pBR322 sequence which replaced the A block sequence was not protected. In contrast, the competition experiment in Figure 3 showed that the 3′ half of the $tRNA_3^{Leu}$ gene competed for protein(s) responsible for the *a* region footprint of the whole $tRNA_3^{Leu}$ gene. When competitor DNAs were tested against the 3′ half probe, results similar to those of Figure 3 with the complete gene probe were found: the complete gene and the 3′ half competed, while the 5′ half and pBR322 did not (data not shown).

The footprint factor-DNA complex is very stable at low ionic strength (Klemenz *et al.*, 1982). In those experiments, the factor was allowed to bind to the labeled footprint probe ($tRNA_3^{Leu}$ gene), then an excess of unlabeled competitor DNA was added and incubation was continued for various periods of time, before adding DNase I. As the bound factor dissociated from the probe DNA, it was likely to be trapped by binding to the excess of competitor, and therefore would become unable to contribute to the footprint. The results demonstrated that the footprint in both the *a* and *b* regions of the DNA was stable. A similar stability experiment was done to compare a $tRNA_3^{Leu}$ gene probe (Figure 1) with a 3′ half gene probe (Figure 4) at 20°C in standard footprinting buffer

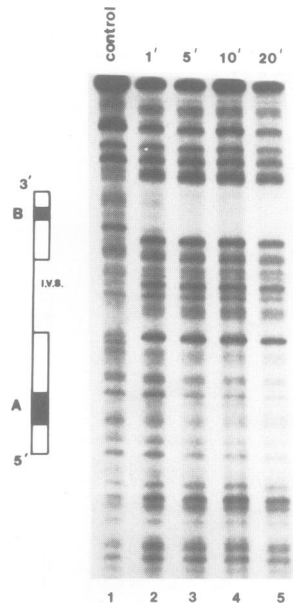

Fig. 6. Kinetics of binding at 20°C. The 341-bp *RsaI/MspI* $tRNA_3^{Leu}$ gene probe was incubated in the absence (**lane 1**) or presence (**lanes 2–5**) of fraction PC 200 for 1 min (**lanes 1, 2**), 5 min (**lane 3**), 10 min (**lane 4**) or 20 min (**lane 5**), followed by DNase I digestion. In this experiment the time of DNase I digestion was reduced from the usual 2 min to 30 s, and the amount of DNase adjusted accordingly. The diagram at the side is described in the legend to Figure 1.

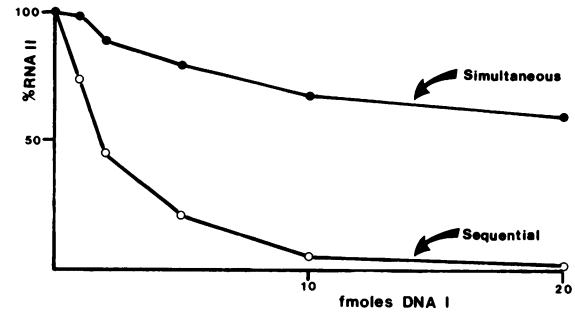

Fig. 7. Transcription competition. **Upper curve**; filled circles: 1 μl of cell-free system was incubated in a total volume of 22.5 μl with 0, 1, 2, 5, 10 or 20 fmol DNA I (pJD 137; $tRNA_3^{Leu}$) and 20 fmol DNA II (pY*SUP*6, $tRNA^{Tyr}$). The total DNA in each reaction mixture was 170 ng, the difference being made up by pBR322. Following a 20 min incubation, 2.5 μl of mixed ribonucleoside triphosphates was added and incubation was continued for an additional 60 min. **Lower curve**; open circles; 1 μl of cell-free system was incubated in a volume of 20 μl with 0, 1, 2, 5, 10 or 20 fmol DNA I. The total DNA in each reaction mixture was 100 ng, the difference being made up with pBR322. After 10 min incubation, 20 fmol of DNA II (2.5 μl, 70 ng) was added. 10 min later, 2.5 μl of mixed ribonucleoside triphosphates was added and incubation continued for a further 60 min. Transcripts were quantitated, as described in Materials and methods. The amount of specific transcript from DNA II was normalized to the reaction without DNA I, defined as 100%.

with 100 mM NaCl and 5% (v/v) dimethyl sulfoxide (DMSO). The probes were pre-incubated with fraction PC 200 for 20 min, then an excess of unlabeled $tRNA_3^{Leu}$ DNA was added as competitor, and stability was tested as just described. Dissociation of the whole gene-protein and of the 3′ half gene-protein complexes could be followed under these conditions (Stillman and Geiduschek, in preparation): the 3′ half gene complex dissociated (only) about three times faster than the whole $tRNA_3^{Leu}$ gene-protein complex (data not shown).

Two further experiments reinforced the suggestion that the B block region dominates the protein-DNA interaction. (i) Figure 5 shows a footprint experiment using a probe prepared from the 5′ half subclone. The region of DNA protected from DNase I extended across the entire 5′ half of the gene, across the linker and into the pBR322 sequence (lane 4). When we examined the lifetime of the protein-DNA complex using the stability protocol described above, the complex was very unstable (data not shown), contrasting with the properties of the 3′ half gene. Competitor DNA, containing the complete gene or the 3′ half, was able to abolish the footprint (lanes 2, 3); indeed pBR322 DNA was only slightly less efficient as a competitor (data not shown). Thus, the isolated 5′ half gene (about which certain qualifications are stated in the Discussion) bound proteins much less strongly than the isolated 3′ half gene. (ii) When the time course of forming the Leu_3 tRNA gene footprint was examined, the *b* region footprint clearly formed more rapidly than the *a* region (Figure 6). For this experiment, $tRNA_3^{Leu}$ gene probe and protein were mixed at 20°C for varying periods of time before the addition of DNase I, with the results shown. The sequential formation of the *b* region and *a* region footprint might merely be due to the action of two independent proteins (or protein complexes) present at different concentrations and/or binding at different rates. However, the following dilution experiment discouraged that interpretation: fraction PC 200 and $tRNA_3^{Leu}$ gene probe were incubated for 1 min, then the sample was split and one half was diluted 8-fold. Both portions were sampled at intervals for footprinting. No differences in the rates of appearance of the *a* region footprints in the diluted and undiluted samples were seen (data not shown). We tentatively conclude that the *a* region footprint might be slowly generated in already-formed protein-DNA complexes.

Transcription factor

The next experiments show that the PC 200 fraction, which contains the footprint-conferring protein, has a transcription factor activity. The basic element in the demonstration is a previously devised assay (Fuhrman *et al.*, 1984). Polymerase III transcription units form stable complexes with accessory proteins which remain bound during repeated rounds of transcription by RNA polymerase III. When DNA templates are in excess, transcriptional activity is entrained to those transcription units which bind the limiting accessory protein. Under these circumstances, if two kinds of polymerase III-transcribed genes are added in sequence to a cell-free system, transcription can be preempted by the first-added DNA (Bogenhagen *et al.*, 1982; Gottesfeld and Bloomer, 1982; Schaack *et al.*, 1983; Fuhrman *et al.*, 1984; Lassar *et al.*, 1983). A protein fraction which provides the limiting accessory protein(s) to such a mixture should rescue the transcription of the second DNA template. The rescue assay was used in the partial purification of a HeLa cell transcription factor binding specifically to regions of the adenovirus 2 VA I gene and other polymerase III-transcribed genes (Fuhrman *et al.*, 1984).

Preemptive transcription complex formation can also be shown with the *S. cerevisiae* cell-free system of Klekamp and Weil (1982). For the experiment shown in Figure 7, DNA I was plasmid pJD137 DNA, containing the $tRNA_3^{Leu}$ gene and DNA II was plasmid pY*SUP*6 DNA, containing the $tRNA^{Tyr}$ gene. When the two templates were added simultaneously,

Table I. Transcriptional competition by $tRNA_3^{Leu}$ complete gene or half gene plasmids

DNA I	Percent transcripts from DNA II (*SUP6* gene)	
	Simultaneous	Sequential
pBR322	100	100
Complete Leu_3 tRNA gene	51	2
3′ half Leu_3 tRNA gene	68	55
5′ half Leu_3 tRNA gene	98	92

The simultaneous and sequential protocols described in Figure 7 were used. The total amount of DNA was 500 ng for DNA I; 25 fmol of pJD137 ($tRNA_3^{Leu}$), pJD 5′ half or pJD 3′ half was used. DNA II was 25 fmol, p*YSUP6* ($tRNA^{Tyr}$, 100 ng).

competition between promoters occurred (Figure 7, closed symbols). When sufficient DNA I was added before DNA II, transcription of the second template was blocked (Figure 7, open symbols). To test the PC 200 fraction for transcription factor activity, we incubated it with DNA II before adding to the cell-free system, which had pre-formed preemptive complexes with DNA I. The PC 200 fraction proved unable to restore the limiting factor sequestered by DNA I (data not shown). We conclude that the limiting component of the yeast cell-free system was not in the footprint-conferring PC 200 fraction.

An alternative approach was suggested by the binding of footprint factor to the 3′ half of the $tRNA_3^{Leu}$ gene (Figure 4), which is not transcribed under the conditions of assay (data not shown). Following simultaneous addition, transcription of the $tRNA^{Tyr}$ gene (p*YSUP6* DNA) was competed by a complete $tRNA_3^{Leu}$ gene or its 3′ half but not its 5′ half (Table I). Competition by 3′ halves of other polymerase III-transcribed genes has been noted (Kressmann *et al.*, 1979; Guilfoyle and Weinmann, 1981; Lassar *et al.*, 1983; Sharp *et al.*, 1983). However, the 3′ half subclone was unable to form a preemptive (or, more precisely in this case, preventive) complex following sequential addition (Schaack *et al.*, 1983). We interpreted these results to show that the 3′ half $tRNA_3^{Leu}$ gene did not bind the limiting factor tightly and thus could not preempt, but was able to compete by binding another, non-limiting factor. We reasoned that a sufficiently large amount of 3′ half $tRNA_3^{Leu}$ plasmid might block the transcription of DNA II in a sequential addition protocol and that this inhibition might be rescued by the footprint-conferring PC 200 fraction. To assess the transcription factor activity of the PC 200 fraction, its effect on the complete cell-free system was tested (Figure 8A, scheme 1, lanes 1–4): transcription of the *SUP6* gene was little affected at these concentrations of PC 200, but changed processing affected the distribution of radioactivity between two tRNA precursor bands. When the cell-free system was pre-incubated with 115 fmol of the 3′ half $tRNA_3^{Leu}$ gene plasmid (scheme 2), the transcription of subsequently added whole $tRNA_3^{Leu}$ gene plasmid (29 fmol) was almost completely blocked (cf. Figure 8A, lane 5, with lane 1). The inhibition could be relieved by fraction PC 200 (Figure 8A, lanes 6–8). A simpler but conceptually less direct experiment (scheme 3) also provided a transcription factor assay. Here, all components were pre-incubated before the addition of ribonucleoside triphosphates. Once again, the transcription of the *SUP6* gene was almost completely inhibited by the 3′ half gene (Figure 8A, lane 9) and the PC 200 fraction restored activity (lanes 10–12). The quantitative analysis of this experiment is shown in Figure 8B, with each series normalized to its control (lanes 2–4 of Figure 8A were normalized to lane 1, lanes

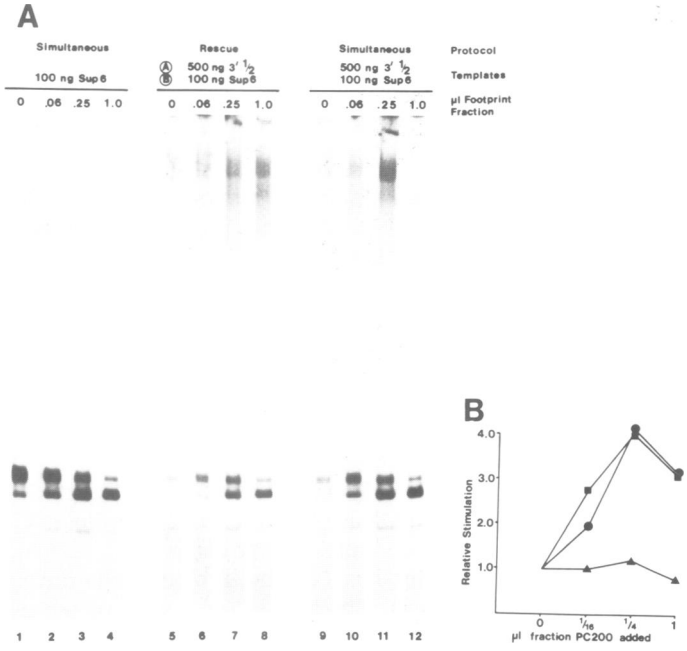

Fig. 8. Transcriptional rescue. **(A) Lanes 1–4** (*Scheme 1*; control, simultaneous):

Cell free system
SUP6 DNA (100 ng) —20 min→ rNTP —60 min→ Analyze
PC 200 fraction ([α-^{32}P]GTP)

1 μl of cell-free system was incubated in 20 μl with 100 ng p*YSUP6* DNA and none **(lane 2)**, 1/16 μl **(lane 2)**, 1/4 μl **(lane 3)**, or 1 μl **(lane 4)** of the PC 200 footprint fraction. After 20 min, 5 μl of mixed ribonucleoside triphosphates was added and incubation was continued for sixty min.
Lanes 5–8 (*Scheme 2*; rescue, sequential):

Cell-free system
A 3′ half DNA (500 ng) —10 min→ mix —10 min→ rNTP → Analyze
B *SUP6* DNA (100 ng) —10 min→ mix
PC 200 fraction ([α-^{32}P]-GTP)

1 μl of cell-free system was incubated with 500 ng of pJD 3′ half gene DNA in a volume of 10 μl. In separate tubes, 100 ng of p*YSUP6* DNA was incubated in 10 μl with none **(lane 5)**, 1/16 μl **(lane 6)**, 1/4 μl **(lane 7)**, or 1 μl **(lane 8)** of the PC 200 fraction. After 10 min, the 10 μl of cell-free system with 3′ half gene DNA was mixed with the PC 200 fraction with p*YSUP6* DNA. After 10 more min, RNA synthesis was started by adding 5 μl of mixed ribonucleoside triphosphates and continued for 60 min.
Lanes 9–12 (*Scheme 3*; rescue, simultaneous):

Cell-free system
3′ half DNA (500 ng)
SUP6 DNA (100 ng) —20 min→ rNTP —60 min→ Analyze
PC 200 fraction ([α-^{32}P]GTP)

Identical with **lanes 1–4**, except that 500 ng pJD 3′ half DNA, 100 ng p*YSUP6* DNA, cell-free system and varying quantities of fraction PC 200 were pre-incubated together before addition of ribonucleoside triphosphates. **(B)** Quantitation. For each sample, in each series, the amount of specific transcription product **(lanes 2–4, ▲; 6–8, ■; 10–12, ●)** was normalized to the corresponding sample without added PC 200 fraction **(lanes 1, 5** or **9**, respectively).

6–8 to lane 6, lanes 10–12 to lane 9). We interpret the experiment as indicating that the 3′ half gene depleted the cell-free system of a transcription factor which could be supplied by the PC 200 fraction.

Discussion

A yeast fraction which contains one or more proteins binding to interior regions of tRNA genes has previously been identified. We have shown here that this fraction also has a transcription factor activity. The definitive identification of the DNA binding activity with the transcription activity requires complete purification, but the correspondence of the footprinting activity to the A and B block promoter regions strongly implies a direct role in promoter recognition. Most tRNA genes have their A and B block promoter segments separated by 30–40 nucleotides (Sprinzl and Gauss, 1982); one such gene ($tRNA^{Gln}$; Klemenz *et al.*, 1983, Figure 2) is entirely protected from DNase I by the PC 200 protein. The $tRNA_3^{Leu}$ gene, which contains a large intervening sequence and a large variable loop, has its A and B blocks separated by 75 nucleotides (Figure 1). The greater separation of the two promoter regions has made it easier to distinguish and characterize protein binding to the separated promoter segments.

The transcription factor interacts with three large regions of the $tRNA_3^{Leu}$ gene in distinguishable ways. (i) Binding to the posterior (*b*) segment of the gene occurs rapidly. Both DNA strands are protected (Figures 1 and 4) as previously shown (Klemenz *et al.*, 1982). Protection of ~25 bp of non-coding strand DNA covering the B block, is almost total. The flanking, unprotected DNA sites are separated by ~40 bp; the limitations of DNase I as the probing reagent make it difficult to be much more precise about the boundaries of the protein covering the *b* region. However, there is bound protein located close to the transcriptional termination site of this gene. (ii) The *i* region of the footprint presents a more complex picture. Protected and unprotected sites alternate (Figure 1B). Such a pattern might arise from protein binding to, or occluding, one side of a DNA helix, but more complex structures involving some unwinding or kinking can be imagined. The intervening sequence (base pairs 39–70) comprises most of the *i* region. (iii) The *a* region footprint covers the anterior portion of the gene, including the A block and extending from around the transcriptional start site downstream for 35 bp. This segment of the footprint is distinguished from the rest in that protection is quantitatively less complete and appears more slowly. Other experiments (Stillman *et al.*, in preparation) suggest that the DNase protection of the *a* region is more salt-sensitive than that of the *i* and *b* regions.

Evidence that the *a* region of the $tRNA_3^{Leu}$ gene is independently, albeit weakly, able to bind protein comes from Figure 5, in which a 5′ half gene probe was examined. However, for the time being, we interpret this result cautiously. Whereas the footprint on the 3′ half of the $tRNA_3^{Leu}$ gene appears to be identical with the *b* region of the complete $tRNA_3^{Leu}$ gene (Figure 4), the footprint of the 5′ half gene is not identical with the complete *a* region of the gene (compare lane 5, Figure 5 and lane 4, Figure 4) but extends across half of the intervening sequence into pBR322. In length, this protected region is the sum of the *a* and *b* regions (cf. $tRNA^{Gln}$, Klemenz *et al.*, 1982). In fact, this construction introduces a sequence which is partly homologous to the B block, provided by the linker and the pBR322 sequences. Thus, it is possible that the weak interaction seen here is, in part, due to extraneous, adventitiously introduced, DNA sequence. This 5′ half subclone is transcriptionally active *in vitro* (Raymond and Johnson, 1983, and data not shown), although it is unable to form stable (preemptive) transcription complexes or to compete against a complete *SUP6* tRNA gene (Table I). Others have also constructed transcriptionally active 5′ half genes or genes in which the A and B blocks are separated by very large distances (Carrara *et al.*, 1981; Sharp *et al.*, 1981; Larson *et al.*, 1983). Nevertheless, further work, with different gene constructions, will be required to see whether the reservations stressed here are necessary.

We surmise that the binding of protein to the *a* and *b* regions is connected, with the *b* region exertin a dominant effect. This is indicated by the competition experiment (Figure 3) in which the 3′ half $tRNA_3^{Leu}$ gene was able to form a stable complex and competed for all of the $tRNA_3^{Leu}$ gene footprint. The dominance of the 3′ half of the gene in binding is reminiscent of transcriptional competition experiments (Kressmann *et al.*, 1979; Guilfoyle and Weinmann, 1981; Larson *et al.*, 1983; Sharp *et al.*, 1983).

Our observations on DNA-protein interaction are compatible with an attractive speculation: that the protein(s) binding to the *a* and *b* regions interact, with the *b* region binding first (Figure 6). If a single multiprotein complex were involved, then the *a* region binding might be an intramolecular event. Alternatively, binding to the *a* and *b* regions might be independent and rapid, but subsequent interactions between the two protein complexes might be required in order to stabilize the interactions of the *a* region with protein. A single protein complex that binds to A and B block-containing regions of polymerase III genes, which are separated by variable distances, must be able to accommodate variable lengths of relatively stiff DNA at a free energy cost that must be kept within certain limits if the transcription complex is to be stable. In that connection, analyzing how the *i* segment of $tRNA_3^{Leu}$ gene bends or folds in its interactions with the binding protein(s) should be informative.

Hall and collaborators (1982) suggested a model involving the single DNA strands of the A and B blocks in a tRNA-like structure. That specific conjecture is now known not to be correct (Newman *et al.*, 1983) but their original focus on the 3-dimensional structure of promoters should continue to influence thinking about these remarkable, small transcription units, which are associated with specific DNA-binding proteins over most of their length. At least some of these interactions persist during transcription, with the RNA chain-elongating polymerase III guided along the template DNA strand over, or past, the DNA-protein complex.

Materials and methods

Materials

Restriction endonucleases (New England Biolabs or Bethesda Research Labs) were used under the conditions specified by the suppliers. Restriction endonuclease *Eco*RI and T4 polynucleotide kinase were the generous gifts of T. Elliott and S. Brennan, respectively. DNase I (RNase-free) was purchased from Miles Laboratories, calf intestinal alkaline phosphatase from Boehringer, DMSO (Spect-AR) from Mallinckrodt, ribonucleoside triphosphates from P.L. Biochemicals, [α-^{32}P]GTP (400 Ci/mmol), from Amersham, and [α-^{32}P]ATP (crude, carrier-free) from ICN.

Plasmids

The following plasmids were used: YEp13($tRNA_3^{Leu}$; Broach *et al.*, 1978), pJD137 ($tRNA_3^{Leu}$; Johnson *et al.*, 1980), p*YSUP6* ($tRNA^{Tyr}$; Johnson and Abelson, 1983) and pJD 5′ half and pJD 3′ half ($tRNA_3^{Leu}$ subclones depicted

in Figure 2; Raymond and Johnson, 1983). Plasmids were purified as described by Kassavetis *et al.* (1983).

Footprinting

The phosphocellulose protein fraction (here called PC 200) which was used for footprinting was prepared as previously described (Klemenz *et al.*, 1982), except that the storage buffer contained 0.1 M NaCl instead of 0.05 M $(NH_4)_2SO_4$. 5′ end-labeled DNA probes were prepared as described (Klemenz *et al.*, 1982). In a footprint assay 1 μl (~10 μg protein) PC 200 fraction, 1–2 fmol labeled DNA probe (5000–10 000 c.p.m., Cerenkov) and 50 ng unlabeled pBR322 carrier DNA was incubated at 20°C for 20 min in 20 μl of buffer FP (20 mM Tris HCl, pH 7.5/100 mM NaCl/7 mM $MgCl_2$/1.25 mM dithiothreitol/7% (v/v) glycerol/5% (v/v) DMSO) containing 5 μg/ml bovine serum albumin (BSA). 10–40 ng of DNase I, which had been diluted shortly before use from a 1 mg/ml stock solution with buffer FP containing 25 μg/ml BSA and 0.5 mM $CaCl_2$, was added in 2 μl, and the digestion was allowed to proceed at 20°C for 2 min. The reaction was stopped by adding 25 μl of 10 mM Tris HCl, pH 8.0/20 mM EDTA/0.2% SDS/200 μg/ml sheared calf thymus DNA/250 μg/ml glycogen and boiled for 3 min. Samples were extracted with phenol:chloroform, and precipitated with isopropanol. DNA fragments were separated on thin 6% polyacrylamide gels containing 8 M urea (Sanger and Coulson, 1978). Gels were exposed wet to flashed Cronex film (Du Pont) using intensifier screens. DNA sequencing (G-specific) reactions were done according to Maxam and Gilbert (1980).

In vitro transcription

The *S. cerevisiae* cell-free system used for transcription was prepared as described by Klekamp and Weil (1982) by A. Voronova, except that the cells [strain 20 B-12, carrying the pep 4-3 mutation (Zubenko *et al.*, 1983), from E.W. Jones, via J. Abelson] were broken with an Eaton press. The transcription system was characterized according to Fowlkes and Shenk (1980) to determine the optimal template and total DNA concentrations. Reaction volumes are given in the figure legends. The initial incubation of proteins with DNA and subsequent additions of DNA were done in buffer FP. When appropriate, protein fraction PC 200 was added in a volume of 1 μl; dilutions of the PC 200 fraction were made in FP buffer with 50 μg/ml BSA. To start RNA synthesis, nucleoside triphosphates (NTP) were added to a final concentration of 0.5 mM for ATP, CTP, UTP, and 0.025 mM for [α-^{32}P]GTP (sp. act., 5 Ci/mmol). Sixty minutes later, reactions were terminated, and samples were processed for electrophoresis on 10% polyacrylamide/8 M urea gels, as described for footprinting. For quantitative analysis, autoradiographs were used to localize the transcription products, which were excised from the gel and counted by liquid scintillation.

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