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Structural features of yeast tRNA genes which affect transcription factor binding

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Transcription of yeast tRNA genes *in vitro* requires, in addition to RNA polymerase III, two accessory factors which are resolved by ion-exchange chromatography. One of these transcription factors (factor C) binds to tRNA genes. The stability of factor C-tRNA gene complexes is gene-dependent: the $tRNA^{Arg}_{AGG}$ gene forms a highly stable complex while $tRNA_3^{Leu}$ and $tRNA^{Tyr}$ gene complexes are unstable under our standard assay conditions. To determine how differences in tRNA gene structure affect factor C binding, mutant $tRNA^{Tyr}$ genes, internally deleted $tRNA_3^{Leu}$ genes and hybrid transcription units containing both $tRNA^{Tyr}$ and $tRNA_3^{Leu}$ segments were compared in their abilities to stably bind factor C. Sequence changes in either of the two highly conserved promoter elements (A block and B block) affect factor C complex stability. Changes towards the consensus sequence increase complex stability while changes away from the consensus sequence drastically reduce stability. Also, the distance separating the A and B blocks affects complex stability; 34–53 bp gives highest stability. These results indicate that the stable binding of transcription factor C to tRNA genes involves interactions with both A block and B block sequences.

Key words: transcription factor/RNA polymerase III/tRNA/yeast

Introduction

Fractionation of human (Segall *et al.*, 1980), frog (Shastry *et al.*, 1982) and yeast (Klekamp and Weil, 1982) cell extracts has revealed that at least two protein factors are required in addition to RNA polymerase III for transcription of tRNA genes *in vitro*. The regions of the DNA template essential for transcription initiation are the conserved A and B block sequences which encode the dihydro U and TΨ arms of tRNA molecules, respectively (Hall *et al.*, 1982). When tRNA genes are incubated with their transcription factors in the absence of RNA polymerase III, stable pre-initiation complexes are formed (Lassar *et al.*, 1983; Fuhrman *et al.*, 1984). Such complexes, which were first observed in the case of *Xenopus* 5S rRNA genes (Bogenhagen *et al.*, 1982), are detected by the ability of a tRNA gene to sequester limiting transcription factor(s) and thereby inhibit transcription of a second tRNA gene added to the *in vitro* reaction at a later time.

Stable pre-initiation complexes provide an experimental tool for identifying the individual DNA-protein interactions which direct tRNA gene transcription initiation. Previous studies have shown that stable complex formation depends on both A and B block sequences (Schaack *et al.*, 1983) and is inhibited if these promoter elements are separated by abnormally long distances (Dingermann *et al.*, 1983). These experiments employed crude cellular extracts, making it difficult to identify and study sequence-specific interactions between individual transcription factors and the tRNA genes involved. In two instances, stable complex formation has been observed with partially purified transcription factors. Ruet *et al.* (1984) found that fraction F_2 of their yeast *in vitro* transcription system formed stable pre-initiation complexes with the yeast $tRNA_2^{Ser}$ and $tRNA_3^{Glu}$ genes. Lassar *et al.* (1983) observed that their factor C from HeLa cells formed a stable pre-initiation complex with the adenovirus VA_I RNA gene but required an additional fraction to form a stable complex with the *Xenopus* $tRNA_1^{Met}$ gene.

We have utilized a fractionated yeast polymerase III transcription system to explore the interactions of separated transcription factors with tRNA genes. One of our fractions contains a transcription factor (factor C) which binds to tRNA genes. The stability of binding of factor C to different tRNA genes is variable. Using a variety of natural and manipulated genes, we have identified several of the structural determinants of tRNA genes which influence the stable binding of this RNA polymerase III transcription factor.

Results

The yeast cell-free extracts used to transcribe tRNA genes *in vitro* were prepared and fractionated essentially as described by Klekamp and Weil (1982). Chromatography on DEAE-Sephadex yielded three distinct fractions which were required to reconstitute *in vitro* transcription activity for all tRNA genes tested (data not shown). One of these fractions contained RNA polymerase III. The two non-polymerase-containing fractions were designated B and C to be consistent with the terminology of Roeder (Lassar *et al.*, 1983). We next sought to determine which fractions contained factors required to form stable pre-initiation complexes with tRNA genes.

Protein factors required for stable complex formation

The formation of stable pre-initiation complexes between transcription factors and tRNA genes was assayed as follows. A plasmid containing a tRNA gene (gene 1) was pre-incubated with a limiting amount of the fraction or fractions to be tested. A plasmid containing a different tRNA gene (gene 2) was then added along with all other components required for the *in vitro* transcription reaction. The two genes were added in equimolar amounts. After further incubation, the RNA products were analyzed by gel electrophoresis. A stable complex formed between the limiting transcription factor and gene 1 will preclude the transcription of gene 2. Figure 1 shows the results of such an experiment; gene 1 was a $tRNA^{Tyr}$ gene (*SUP4*), gene 2 a $tRNA_3^{Leu}$ gene (*sup53*$^+$). Pre-incubations were carried out at both 0° and 25°C. In neither case where fraction B or fraction C alone was pre-incubated with the $tRNA^{Tyr}$ gene was transcription of the $tRNA_3^{Leu}$ gene inhibited (lanes 3,4,7,8). However, when both fractions B and C were present during pre-incubation, either at 0°C

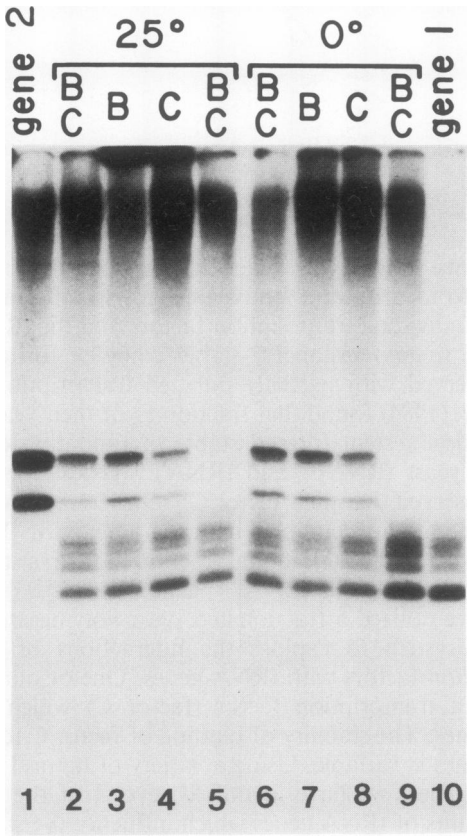

Fig. 1. Formation of stable pre-initiation complexes. 75 ng of plasmid pAsup4-4 (gene 1, $tRNA^{Tyr}$) were incubated for 10 min at the indicated temperatures with the indicated protein fraction(s) (2 μl fraction C and/or 4 μl fraction B). 100 ng of plasmid CV13 (gene 2, $tRNA_3^{Leu}$) were then added, followed by the other fractions and components needed for *in vitro* transcription reactions [final amounts in 20 μl:2 μl fraction C, 4 μl fraction B, 1 μl polymerase III-containing fraction, 6.5 mM $MgCl_2$, 110 mM KCl, 20 mM Hepes (pH 7.9), 200 μg/ml BSA; 500 μM ATP, GTP, CTP; 50 μM [α-^{32}P]UTP (8 Ci/mmol), 13% glycerol]. Reactions were then carried out as described in Materials and methods. For the reactions of **lanes 2** and **6**, gene 2 was included in the first incubation along with gene 1. The outside gel lanes show the RNA products of reactions containing only the gene indicated.

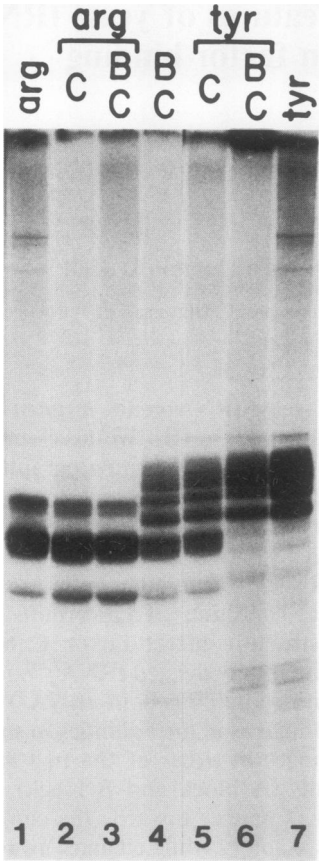

Fig. 2. The $tRNA^{Arg}_{AGG}$ gene requires only fraction C to form a stable pre-initiation complex. 60 ng pSarg ($tRNA^{Arg}_{AGG}$, **lanes 2** and **3**) or pAsup4-4 ($tRNA^{Tyr}$, **lanes 5** and **6**) were incubated at 25°C for 5 min with 2 μl fraction C and with or without 5 μl fraction B, as indicated. 60 ng pAsup4-4 (**lanes 2** and **3**) or pSarg (**lanes 5** and **6**) was then added, followed 5 min later by remaining transcription reaction components. For **lane 4**, both genes were included in the pre-incubation. Reactions of **lanes 1** and **7** contained only $tRNA^{Arg}_{AGG}$ or $tRNA^{Tyr}$ plasmids, respectively. Details of pre-incubation and *in vitro* transcription conditions are given in Materials and methods.

(lane 9) or 25°C (lane 5), subsequent transcription of the $tRNA_3^{Leu}$ gene was precluded. The limiting transcription factor had apparently become stably associated with the $tRNA^{Tyr}$ gene during pre-incubation and only pre-$tRNA^{Tyr}$ was produced during the subsequent transcription reaction. The preponderance of $tRNA^{Tyr}$ product was not simply due to a preferential binding of transcription factor to the $tRNA^{Tyr}$ gene, because when both genes were present during pre-incubation they were transcribed approximately equally (lanes 2,6). It is not possible to say whether fraction B or fraction C contained the limiting factor, but clearly both are required to form a stable transcription complex with the $tRNA^{Tyr}$ gene under these conditions.

Quite different results were obtained when the pre-incubation experiment was carried out with a $tRNA^{Arg}_{AGG}$ gene as gene 1 (Figure 2). Pre-incubation of the $tRNA^{Arg}_{AGG}$ gene with fraction C alone was sufficient to preclude transcription of the second gene, in this case the $tRNA^{Tyr}$ gene (lane 2). This result indicates that fraction C contains the limiting factor and also that this factor is capable of forming a stable complex with the $tRNA^{Arg}_{AGG}$ gene in the absence of fraction B. Lanes 5 and 6 of Figure 2 confirm that both fractions B and C are required to form such a stable transcription complex with the $tRNA^{Tyr}$ gene. For ease of discussion we will henceforth refer to the limiting transcription factor in fraction C as 'factor C'.

Stability of the factor C-$tRNA^{Tyr}$ gene complex

The apparent inability of factor C to form a stable complex with the $tRNA^{Tyr}$ gene in the absence of fraction B was investigated further by varying several parameters of the basic pre-incubation protocol. The first variable tested was the length of time during which both genes were present with fraction C in the absence of fraction B. The $tRNA^{Tyr}$ gene was first pre-incubated with fraction C, then $tRNA^{Arg}_{AGG}$ gene was added and the pre-incubation continued. At various times after $tRNA^{Arg}_{AGG}$ gene addition, fraction B, RNA polymerase III and nucleotide triphosphates were added to initiate the transcription reactions. The results (Figure 3A) show that when fraction B was added simultaneously with the $tRNA^{Arg}_{AGG}$ gene, $tRNA^{Arg}_{AGG}$ transcription was prevented (lane 2); however, as the length of time between $tRNA^{Arg}_{AGG}$ gene addition and fraction B addition increased, so did the proportion of $tRNA^{Arg}_{AGG}$ product (lanes 3–5). The fact that adding fraction B simultaneously with the second gene is suf-

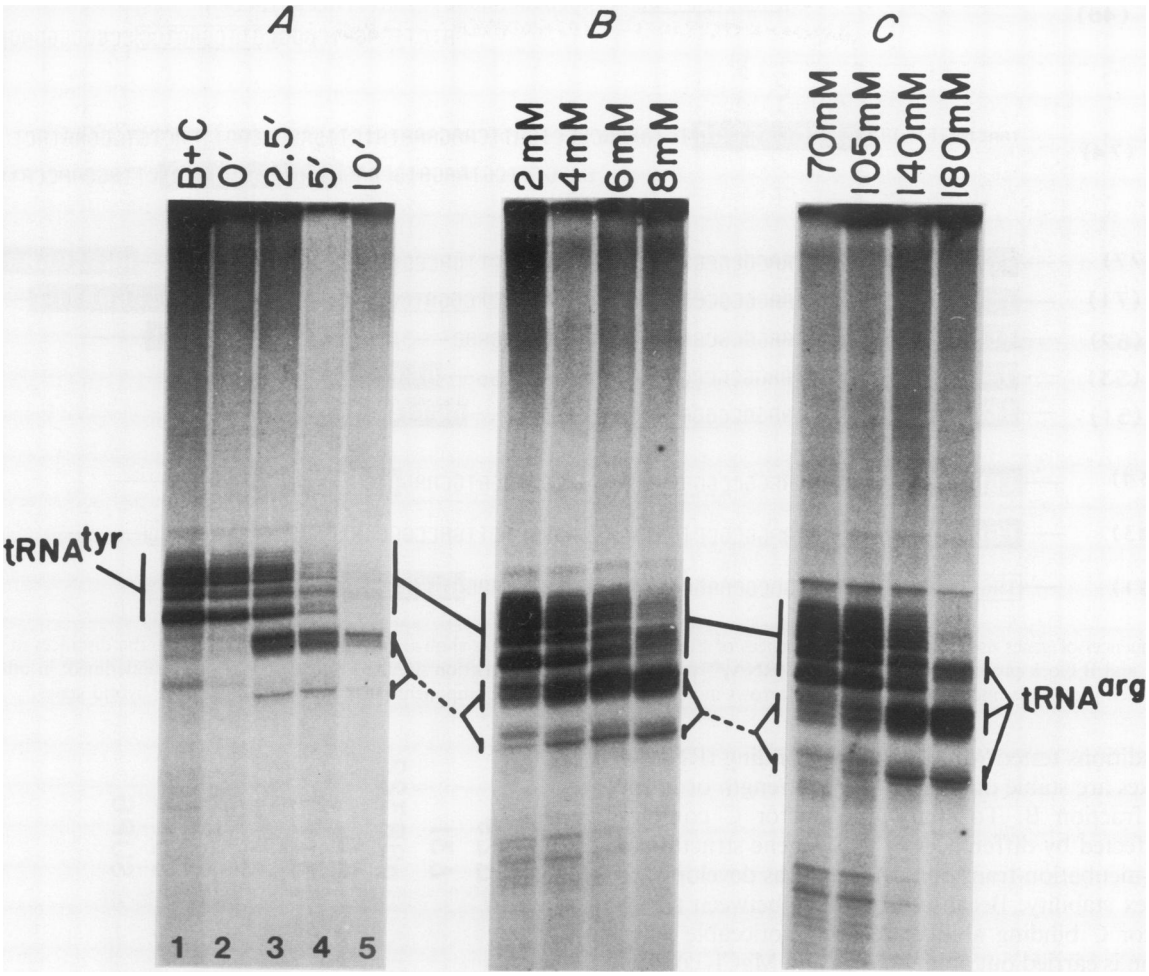

Fig. 3. Stability of factor C-tRNA gene complexes. Each reaction consisted of a pre-incubation step followed by an *in vitro* transcription reaction. For the pre-incubations, fraction C was first incubated with 60 ng pAsup4-4 ($tRNA^{Tyr}$ gene) for 5 min at 25°C, then 60 ng pSarg ($tRNA^{Arg}_{AGG}$ gene) was added and the pre-incubation continued at 25°C until remaining reaction components were added to start transcription. **(A)** Pre-incubation buffer contained 5 mM $MgCl_2$ and 150 mM KCl. The length of pre-incubation after addition of pSarg and the start of the transcription reaction was varied as indicated. For the 0 min reaction, pSarg was added simultaneously with remaining transcription reaction components. In the reaction of **lane 1**, $tRNA^{Tyr}$ gene was mixed with both fraction B and fraction C before pSarg addition; otherwise, conditions were identical to the 10 min reaction. **(B)** Conditions and timing were as for the 5 min reaction of part **A** except the $MgCl_2$ concentration of the pre-incubation buffer was varied as indicated. **(C)** Conditions as in part **B** except the $MgCl_2$ concentration of the pre-incubation buffer was kept constant (5 mM) and the KCl concentration was varied as indicated. Details of the pre-incubation-transcription protocol are given in Materials and methods.

ficient to preclude transcription of the second gene indicates that factor C had already been sequestered with the $tRNA^{Tyr}$ gene by the end of pre-incubation and that fraction B is not required for the formation of a complex between factor C and the $tRNA^{Tyr}$ gene. Fraction B is required to stabilize the interaction between factor C and $tRNA^{Tyr}$ gene, because in its absence factor C-$tRNA^{Tyr}$ gene complexes dissociate and factor C-$tRNA^{Arg}_{AGG}$ gene complexes form. Both genes form a complex with factor C but, under these conditions, the $tRNA^{Arg}_{AGG}$ gene complex is stable while the $tRNA^{Tyr}$ gene complex is not.

We next sought to determine if experimental conditions existed under which $tRNA^{Tyr}$ gene-factor C complexes would be stable. The experiments described in Figure 3B and C demonstrate the ionic dependence of complex stability. In both cases the $tRNA^{Tyr}$ gene was pre-incubated with factor C to allow formation of the complex, then the $tRNA^{Arg}_{AGG}$ gene was added. After 5 min to allow redistribution of factor C between the two genes, fraction B and remaining reaction components were added. Conditions favoring complex stability will lead to predominantly $tRNA^{Tyr}$ product, while conditions causing complex instability will lead to predominantly $tRNA^{Arg}_{AGG}$ product. The results show that decreasing $MgCl_2$ concentration (Figure 3B) or KCl concentration (Figure 3C) increases $tRNA^{Tyr}$ gene-factor C complex stability as seen by an increase in the proportion of $tRNA^{Tyr}$ product formed in the subsequent transcription reactions. In fact, when KCl and $MgCl_2$ concentrations during pre-incubation are reduced, to 70 mM and 2 mM, respectively, $tRNA^{Tyr}$ gene-factor C complexes are as stable as those formed in the presence of fraction B (data not shown). The association between factor C and $tRNA^{Tyr}$ plasmid at low ionic strength is due to the presence of the 260-bp $tRNA^{Tyr}$ gene-containing fragment, because incubation of fraction C and pBR322 DNA under these conditions does not lead to significant loss of factor C availability for subsequent $tRNA^{Arg}_{AGG}$ gene transcription (data not shown).

Stability of factor C pre-initiation complexes with $tRNA^{Leu}_3$ genes

The experiments just described demonstrated that factor C is capable of forming complexes of varying stabilities with yeast tRNA genes. Factor C-$tRNA^{Arg}_{AGG}$ gene complexes are stable

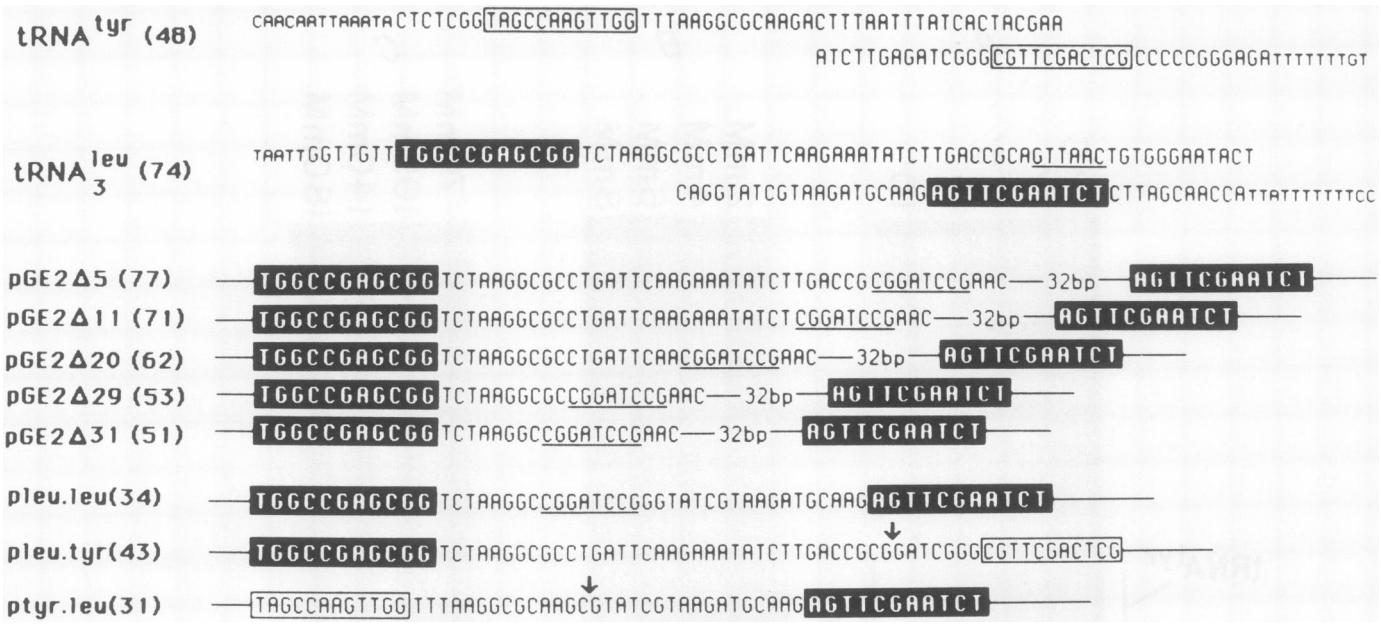

Fig. 4. DNA sequences of genes used in this study. Sequences of the non-coding strands are shown. Numbers in parentheses are the distances in base pairs separating the A and B block promoter elements. In the $tRNA_3^{Leu}$ sequence, the *Hpa*I restriction site used to create deletions is underlined; in other cases, the *Bam*HI linker (when present) is underlined. The vertical arrows indicate the site at which component parts were joined to create hybrid genes.

under all conditions tested while the corresponding $tRNA^{Tyr}$ gene complexes are stable only at low ionic strength or in the presence of fraction B. To study how factor C complex stability is affected by differences in tRNA gene structure, a standard pre-incubation-transcription assay was developed to assess complex stability. Because differences between tRNA genes in factor C binding ability are most noticeable when pre-incubation is carried out at higher salt and $MgCl_2$ concentrations, the various genes to be tested are incubated with factor C in the presence of 5 mM $MgCl_2$ and 150 mM KCl. A second gene is then added and incubation continued with both genes present for 5–10 min; then all remaining transcription reaction components are added. The amount of RNA transcribed from the second gene provides a measure of the instability of the initial complex.

When pre-incubated with factor C under the standard assay conditions, the yeast $tRNA_3^{Leu}$ gene fails to make a stable complex (Figure 5, lane 7). Instability of the complex might be a consequence of deviations from optimal A and B block sequences; alternatively, the longer than average length of DNA separating the A and B blocks of this gene (74 bp) might reduce the stability of factor C binding. To test for an effect of A to B block distance, a set of shortened $tRNA_3^{Leu}$ genes was constructed and tested for the ability to form stable complexes with factor C. Deletions extending in either direction from an *Hpa*I site within the $tRNA_3^{Leu}$ intron region were made by exonuclease digestion and insertion of a synthetic *Bam*HI linker sequence. The resulting deleted $tRNA_3^{Leu}$ genes have A block to B block distances which range from 77 to 34 bp (Figure 4). Measurements of the stability of factor C complexes with these mutant genes and with the $tRNA_3^{Leu}$ gene are shown in Figure 5. Both the naturally-occurring gene and deletion Δ5 (A to B = 77 bp) form highly unstable complexes (lanes 7 and 4). With further deletion (lanes 5,6, 1,2), the stability of the factor C-gene complex increases dramatically, resulting in decreased transcription of the $tRNA_{AGG}^{Arg}$ product and a concomitant increase in accumulation of the shortened $tRNA_3^{Leu}$ product. When A block-B

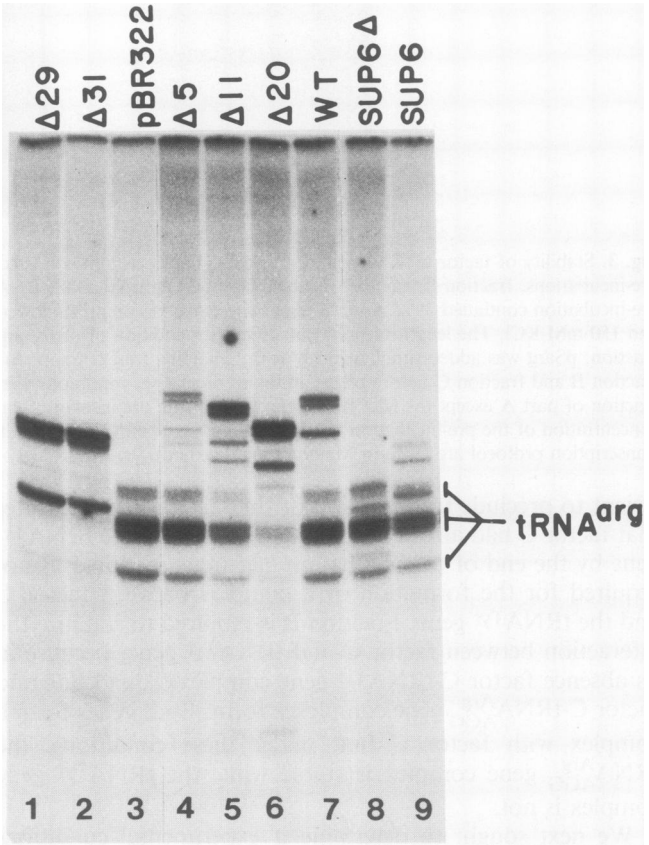

Fig. 5. Effect of internal deletions on factor C complex stability. The following tRNA gene-containing plasmids were tested in the factor C complex stability assay using pSarg ($tRNA_{AGG}^{Arg}$) as the second gene (see Materials and methods): **lane 1**, pGE2Δ29; **lane 2**, pGE2Δ31; **lane 3**, pBR322; **lane 4**, pGE2Δ5; **lane 5**, pGE2Δ11; **lane 6**, pGE2Δ20; **lane 7**, pGE2wt (naturally occurring $tRNA_3^{Leu}$ gene); **lane 8**, pYSUP6Δ32; **lane 9**, pYSUP6. Nucleotide sequences of the deleted $tRNA_3^{Leu}$ genes are given in Figure 4.

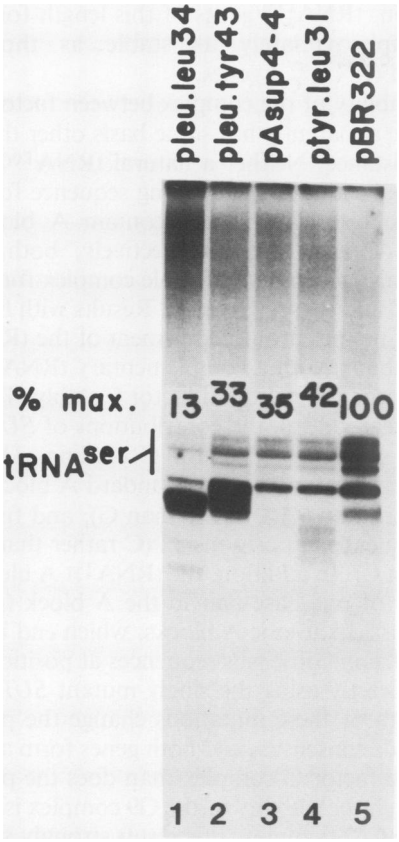

Fig. 6. Stability of factor C complexes of hybrid genes. The plasmids indicated were tested in the factor C complex stability assay using pPM5 ($tRNA^{Ser}_{UCG}$) as the second gene. The reactions were run in duplicate, but only half of the gel autoradiogram is shown. $tRNA^{Ser}_{UCG}$ bands were quantitated by densitometry and the mean of the two determinations is displayed in the figure. The average deviation from the mean for the five determinations was 2%.

block distance is reduced to 53 bp (lane 1) or 51 bp (lane 2), the resulting genes form complexes with factor C that are completely stable under these conditions. The inability of the natural $tRNA^{Leu}_3$ gene to form a stable complex with factor C, therefore, is not due to its A or B blocks *per se*, but rather to the spatial relationship between them.

Effects of A block and B block sequences upon the stability of factor C complexes

Factors other than A block-B block distance are required to explain the inability of $tRNA^{Tyr}$ genes to form stable complexes with factor C. The $tRNA^{Tyr}$ distance of 48 bp is already shorter than the 53 bp distance which allowed for stable complex formation in the $tRNA^{Leu}_3$ gene series. For the *SUP6* $tRNA^{Tyr}$ gene, shortening of this distance to 34 bp by deleting the intervening sequence did not increase complex stability (Figure 5, lanes 8 and 9).

To determine if the inability of $tRNA^{Tyr}$ genes to form stable complexes with factor C could be directly attributed either to A block or B block structure, hybrid genes were constructed by combining the A block from a $tRNA^{Tyr}$ gene and the B block from a $tRNA^{Leu}_3$ gene and *vice versa*. The hybrid genes contained A block-B block distances of 31 and 43 bp, respectively. As a control, an altered $tRNA^{Leu}_3$ gene was constructed which contained an A block-B block distance of 34 bp (Figure 4). Complex stabilities were measured exactly as for Figure 5 except the yeast $tRNA^{Ser}_{UCG}$ gene was used as

Table I. Factor C complex stabilities of mutant *SUP4* genes

Gene 1	Gene 2 ($tRNA^{Arg}_{AGG}$) transcription (mean ± standard deviation)
SUP4	1.00 ± 0.08
G9	0.82 ± 0.13
U10	1.89 ± 0.23
G56	1.98 ± 0.07
C62	0.54 ± 0.07
pBR322	1.49 ± 0.17

The various genes were tested in quadruplicate in the standard stability assay using pSarg ($tRNA^{Arg}_{AGG}$) as the second gene (see Materials and methods). pSarg transcription was quantitated by densitometry and is normalized to that of the *SUP4* reactions. Values <1.0 therefore indicate a factor C complex more stable than the *SUP4* complex, while values >1.0 indicate a less stable complex.

the second gene instead of the $tRNA^{Arg}_{AGG}$ gene, because transcription products from one of the hybrid genes overlapped slightly with those from the $tRNA^{Arg}_{AGG}$ gene. Like the $tRNA^{Arg}_{AGG}$ gene, the $tRNA^{Ser}_{UCG}$ gene forms very stable complexes with factor C (data not shown) and is therefore suitable for this purpose. The two $tRNA^{Leu}_3$/$tRNA^{Tyr}$ hybrid genes form factor C complexes with stabilities more or less equal to that of the natural $tRNA^{Tyr}$ gene and significantly lower than that formed by the shortened $tRNA^{Leu}_3$ gene (Figure 6). Thus the replacement, in a $tRNA^{Leu}_3$ transcription unit, of either the 5′ or 3′ half with a corresponding $tRNA^{Tyr}$ element causes reduced stability of the factor C complex. Both the A block and B block of the $tRNA^{Tyr}$ gene must therefore lack qualities essential for stable binding of factor C.

Aside from the question of complex stabilities, Figure 6 points out a major difference among genes with respect to their template activities. Genes containing the A block of the $tRNA^{Leu}_3$ gene are transcribed more actively than genes containing the A block of the $tRNA^{Tyr}$ gene. Comparison is best made in the case of the natural $tRNA^{Tyr}$ gene and the hybrid gene containing a $tRNA^{Leu}_3$ A block and a $tRNA^{Tyr}$ B block (lanes 2 and 3); both genes have sequestered approximately equal amounts of factor C, yet the hybrid gene clearly produces more RNA. Because these genes differ not only in their A blocks, but also in their upstream flanking sequences, it is impossible to conclude which of these regions is responsible for the reduced activity of the $tRNA^{Tyr}$ gene. The critical difference, however, must lie upstream of the $tRNA^{Tyr}$ B block.

The influence of the $tRNA^{Tyr}$ gene promoter sequence on factor C complex stability was tested directly by employing cloned genes of *SUP4* with single point mutations (Kurjan *et al.*, 1980). The genes G9 and U10, and G56 and C62 carrying single base pair changes in their A and B block sequences, respectively, were chosen for study, because these mutations were previously found to affect *SUP4* promoter activity in a crude extract (Allison *et al.*, 1983). [The mutant genes are named to indicate the identity and position of the base change; i.e., G9 has a G (rather than A) at position 9 (Kurjan *et al.*, 1980).] Results of complex stability assays are given in Table I. Both G9 and C62 genes form complexes with factor C which are more stable than the wild-type gene complex; the stability increase for C62 is quite dramatic. These results support the conclusion from the hybrid gene experiment that neither the A block nor the B block of the natural $tRNA^{Tyr}$

gene is optimal for stable factor C complex formation. The U10 and G56 mutations are totally detrimental to complex stability. Pre-incubation of these mutant $tRNA^{Tyr}$ genes with factor C is less effective than pBR322 pre-incubation in reducing subsequent $tRNA^{Arg}_{AGG}$ gene transcription. No systematic attempt was made to test base pair changes throughout the *SUP*4 gene; however, results from these four mutant $tRNA^{Tyr}$ genes clearly indicate that both A block and B block sequences play a role in the stable binding of factor C.

Discussion

A number of *in vitro* studies with whole cell extract RNA polymerase III transcription systems have shown that stable pre-initiation complexes form between RNA polymerase III accessory factors and both 5S rRNA genes (Bogenhagen *et al.*, 1982) and tRNA genes (Fuhrman *et al.*, 1984; Lassar *et al.*, 1983). The existence of such complexes is inferred from the fact that prior incubation of transcription factors with one gene precludes the transcription of a second gene added later. A limiting factor becomes stably associated with the first gene and is not available for transcription of the second gene (Bogenhagen *et al.*, 1982).

Lassar *et al.* (1983) extended the study of pre-initiation complex formation to a fractionated human cell (HeLa) system consisting of purified RNA polymerase III plus accessory factors A, B and C. *In vitro* 5S rRNA gene transcription required all three fractions in addition to RNA polymerase III, while the polymerase together with fractions B and C sufficed for transcription of both the adenovirus VA_I RNA gene and the *Xenopus* $tRNA^{Met}_1$ gene. With the VA_I RNA gene, factor C alone was sufficient to form a stable complex; however, the $tRNA^{Met}_1$ gene was able to form such a stable complex only if it was incubated together with both fractions B and C prior to the addition of the second template (Lassar *et al.*, 1983). Factor C apparently binds at or near the B block of the adenovirus VA_I RNA gene internal promoter sequence, because a *Bam*HI site in this region is protected from digestion after incubation of the VA_I gene with factor C (Lassar *et al.*, 1983).

In our fractionated yeast RNA polymerase III transcription system two fractions, B and C, are required for polymerase III transcription of all tRNA genes we have tested; however, the requirements for stable pre-initiation complex formation differ from gene to gene. The yeast $tRNA^{Arg}_{AGG}$ and $tRNA^{Ser}_{UCG}$ genes are able to form stable pre-initiation complexes with fraction C alone, whereas the $tRNA^{Leu}_3$ and $tRNA^{Tyr}$ genes form highly stable complexes only when both fractions B and C are present. Our results show that this difference is due directly to differences in the stability of complexes formed between factor C and each of these genes. [We have assumed that fraction C contains only one transcription factor active on tRNA genes, factor C (see below).]

The main objective of our study has been to find the basis in tRNA gene structure for differences in factor C binding affinity. To this end, we created a series of internally deleted $tRNA^{Leu}_3$ genes lacking various amounts of the DNA sequence between the conserved A and B block sequences. The progressive increase in stability of the $tRNA^{Leu}_3$ gene complex with factor C when sequences between the A and B blocks are deleted suggests that it is the long A to B block distance of the natural $tRNA^{Leu}_3$ gene (74 bp) which prevents it from stably binding factor C. A to B block distances in the range of 34–53 bp are, in our experiments, optimal for stable complex formation. $tRNA^{Leu}_3$ genes of this length form factor C complexes approximately as stable as those of the $tRNA^{Arg}_{AGG}$ and $tRNA^{Ser}_{UCG}$ genes.

The low stability of the complex between factor C and the $tRNA^{Tyr}$ gene apparently has some basis other than A block to B block distance. Neither a natural $tRNA^{Tyr}$ gene nor a $tRNA^{Tyr}$ gene lacking its intervening sequence form a stable factor C complex. These genes contain A block-B block distances of 48 and 34 bp, respectively, both within the 34–53 bp range which allows stable complex formation between factor C and $tRNA^{Leu}_3$ genes. Results with hybrid genes indicated that neither promoter element of the $tRNA^{Tyr}$ gene could be combined with a complementary $tRNA^{Leu}_3$ element to produce a gene which bound factor C stably. This led us to examine in greater detail the contributions of *SUP*4 A block and B block sequences to factor C binding. The $tRNA^{Tyr}$ gene sequence deviates from the standard A block consensus sequence at position 9 (A rather than G); and from B block consensus sequence at positions 52 (C rather than G) and 62 (G rather than C). In addition, the $tRNA^{Tyr}$ A block contains an 'insertion' of one base pair in the A block (position 17) relative to most eukaryotic A blocks, which end in AGYGG. The effects of non-consensus sequences at positions 9 and 62 were tested directly using the singly mutant *SUP*4 genes G9 and C62. Both of these mutations change the promoter sequence towards consensus, and both genes form a significantly more stable factor C complex than does the parent *SUP*4 allele. Although the stability of the G9 complex is not as great as that of the C62 complex, this result strongly suggests that deviations of the $tRNA^{Tyr}$ promoter elements from the optimal A and B block sequences are a principal cause for this gene's inability to bind factor C stably. Studies with the yeast $tRNA^{Leu}_3$ and $tRNA^{Tyr}$ mutant genes and the $tRNA^{Leu}_3$/$tRNA^{Tyr}$ hybrid genes all lead to the same conclusion: that both A and B block sequences play a role in the binding of factor C.

We have assumed throughout that the transcription factor activity attributed to factor C derives from a single protein; i.e., that our fraction C contains only one transcription factor. That subsequent chromatography of fraction C on heparin-agarose resolves no additional factors (R.Baker, unpublished observation) supports this assumption, but ultimate proof will only be obtained when factor C is totally purified. Significant progress towards this end has been made by Ruet *et al.* (1984), who have partially purified a yeast transcription factor that they designate τ. Tau factor is probably the same as our factor C; it exhibits chromatographic behavior similar to factor C and, like factor C, forms stable complexes with tRNA genes (Ruet *et al.*, 1984). Ruet *et al.* (1984) estimate that τ factor has a mol. wt. of ~300 000 daltons. A protein of this size is large enough to undergo the interactions we postulate for factor C, i.e., binding simultaneously to two different, variably-spaced sequence elements.

Physical evidence for the binding of a yeast protein to A and B block sequences of tRNA genes is provided by the DNaseI protection (footprinting) experiments of Stillman and Geiduschek (1984). They obtained a yeast protein fraction containing one or more proteins which, as inferred from an ability to confer protection against DNaseI cleavage, bind to the promoter elements of several tRNA genes. In the case of the yeast $tRNA^{Leu}_3$ gene, the protection pattern was most complete in the B block region; protection of A block sequences was observed, but it was less complete, slower to

appear, and more salt sensitive (Stillman and Geiduschek, 1984). The authors suggested that the binding they observed might be due to a single, complex protein interacting with both A and B block sequences (Stillman and Geiduschek, 1984).

The footprinting results just described are clearly consistent with our findings concerning the binding of transcription factor C. Given its chromatographic history (Klemenz *et al.*, 1982), the protein fraction used by Stillman and Geiduschek would be expected to contain our factor C in addition to some proteins of our fraction B. Based on the footprinting results and our current *in vitro* transcription results we would propose that factor C interacts with tRNA gene B block sequences to form an unstable complex. Subsequent interaction with A block sequences is required to complete the formation of the stable pre-initiation complex. Some genes, e.g., $tRNA^{Tyr}$ and $tRNA_3^{Leu}$, require an additional factor (in our fraction B) for stable pre-initiation complex formation. A relative deficiency (or absence) of this factor might explain why, in footprinting experiments with the $tRNA_3^{Leu}$ gene (Stillman and Geiduschek, 1984), protection of A block sequences is only partial.

The differences which we have observed in the ability of various classes of yeast tRNA genes to form stable transcription complexes may reflect a control mechanism operative in yeast cells. Depending upon the intracellular concentration of factor C relative to the number of yeast tRNA genes, the transcriptional output may vary for different yeast tRNA genes. Under conditions of limiting factor C, yeast tRNA genes which lack high affinity factor C binding sequences (such as $tRNA^{Tyr}$ and $tRNA_3^{Leu}$ genes) would be transcribed much less actively than $tRNA^{Ser}_{UCG}$, $tRNA^{Arg}_{AGG}$ genes and other genes resembling them (Ruet *et al.*, 1984). If, on the other hand, sufficient quantities of factor C were available to bind to all yeast tRNA genes, other stages of transcription initiation would become limiting and the relative transcriptional activity of different yeast tRNA genes would shift accordingly. As Brown (1984) has pointed out, the differential effect of such stable transcriptional complexes may be enhanced because of the mutually exclusive effects of transcription factor binding and nucleosome assembly. Shortly after they are chromosomally replicated, those tRNA genes which do not acquire a factor C molecule may instead be packaged as nucleosomes, preventing their activation until the nucleosomes on them are disrupted or segregated in the next replication cycle. Therefore, given the great divergence of factor binding properties amongst yeast tRNA genes, the relative production of different tRNA species may be changed simply by altering the intracellular availability of transcription factor C for tRNA gene transcription.

Materials and methods

Materials

All enzymes used in the construction and analysis of plasmids were purchased from Bethesda Research Laboratories and were used under the conditions recommended by the manufacturer. *Bam*HI linkers were purchased from Collaborative Research, nucleotide triphosphates from P-L Biochemicals, [α-^{32}P]UTP from Amersham, P-11 cellulose phosphate from Whatman, and DEAE-Sephadex A-25 from Pharmacia.

Plasmids

pPM5 containing the yeast $tRNA^{Ser}_{UCG}$ gene (Olson *et al.*, 1981) and the plasmids containing the wild-type and mutant *SUP*4 $tRNA^{Tyr}$ genes (Allison *et al.*, 1983) have been described. Plasmid pSarg containing the yeast $tRNA^{Arg}_{AGG}$ gene (Gafner *et al.*, 1983) on a 0.6-kb *Xho*I-*Sau*3A fragment was constructed by D. Allison in this laboratory. Plasmid pAsup4-4 contains the *SUP*4 $tRNA^{Tyr}$ gene on a 260-bp *Alu*I fragment inserted at the *Bam*HI site of pBR322. pYSUP6 and pYSUP6Δ32 containing the yeast *SUP*6 $tRNA^{Tyr}$ gene with and without its intervening sequence, respectively, were obtained from J.Abelson (Wallace *et al.*, 1978). Plasmid CV13 (YEp13) which contains the yeast *sup*53 $tRNA_3^{Leu}$ gene has been described (Broach *et al.*, 1979; Andreadis *et al.*, 1982).

$tRNA_3^{Leu}$ genes containing internal deletions were obtained by exonuclease (*Bal*31) digestion of genes cleaved at the *Hpa*I site located in their intervening sequence. After addition of synthetic *Bam*HI linkers, a collection of plasmids (pGE2Δn) was obtained, all containing the deleted (or wild-type) gene on a 1.3-kb *Xho*I-*Eco*RI fragment inserted into pBR322 between the *Sal*I and *Eco*RI sites. Deletion endpoints were determined by DNA sequencing (Maxam and Gilbert, 1980).

Construction of hybrid genes was facilitated by the fact that all of the deleted $tRNA_3^{Leu}$ genes contained a unique *Bam*HI site at varying distances from their promoter elements. Thus, either the A block or B block of any of the $tRNA_3^{Leu}$ genes could be removed simply by digestion with *Bam*HI and an appropriate second restriction enzyme and replaced with a fragment derived from the *SUP*4 $tRNA^{Tyr}$ gene. A *SUP*4 B block-containing fragment was obtained by virtue of a *Sau*3A site located 7 bp upstream from the start of the B block sequence. A *SUP*4 A block-containing fragment was obtained from an M13 clone containing 5′-flanking sequences and the first 32 bp of coding sequence of the *SUP*4 gene (D.Allison). Sequences of the hybrid genes were determined by Maxam and Gilbert DNA sequencing.

Details of the construction of the deleted $tRNA_3^{Leu}$ and hybrid gene-containing plasmids as well as restriction maps will gladly be provided upon request.

Superhelical plasmid DNA was prepared for *in vitro* transcription assays by CsCl gradient centrifugation (Clewell, 1972).

Preparation and fractionation of in vitro transcription extracts

Yeast extracts were prepared and fractionated by the methods of Klekamp and Weil (1982) with the following modifications. Extracts were prepared from 20–35 g batches of *S. cerevisiae* RNase$^-$3 cells (Littlewood *et al.*, 1971) broken in a Braun homogenizer. Buffers were identical except KCl was substituted for NaCl. For the phosphocellulose chromatography, proteins were applied in buffer containing 0.1 M KCl and eluted in buffer containing 0.5 M KCl. After dialysis to reduce the KCl concentration to 0.1 M, this fraction was applied to DEAE-Sephadex. Step fractions were obtained by raising the KCl concentration to 0.25 M, then 1.0 M. Our fraction B, fraction C and polymerase III fraction correspond, respectively, to fractions d, e and f of Klekamp and Weil.

In vitro transcription reactions

The standard transcription reaction contained in a total volume of 25 μl; 5 μl fraction B, 2 μl fraction C, 1 μl partially purified RNA polymerase III and 100–200 ng of tRNA gene-containing plasmid DNA. Final concentrations of buffer components were: 20 mM Hepes, pH 7.9; 5 mM $MgCl_2$; 125 mM KCl; 0.04% Nonidet P-40 (NP-40); 200 μg/ml bovine serum albumin (BSA); 6% glycerol; 500 μM each of GTP, ATP and CTP; 50 μM [α-^{32}P]UTP (8–12 Ci/mmol). Reactions were incubated at 25°C for 30 min and terminated as described (Allison *et al.*, 1983). The ethanol-precipitated RNA products were dissolved in 7 μl loading buffer (10 mM sodium acetate, pH 5, 8 M urea, 0.05% each of bromphenol blue and xylene blue cyanol) and resolved by electrophoresis on a 10% polyacrylamide gel in Tris-borate buffer containing 7 M urea (Koski *et al.*, 1982). Gels were dried and used to expose Kodak XRP film. To quantitate bands, films were scanned on a Helena Laboratories 'Quick Scan' densitometer. Film response was linear under the exposure conditions employed.

Factor C complex stability assay

20 fmol (60–100 ng) of plasmid containing the gene to be tested was incubated for 5 min at 25°C with 2 μl of fraction C in 10 μl total volume of buffer containing 10 mM Hepes, pH 7.9, 5 mM $MgCl_2$, 150 mM KCl, 0.1% NP-40, 500 μg/ml BSA, and 4% glycerol. Differences in total DNA concentration (due to varying plasmid mol. wts.) were balanced by the addition of pBR322 plasmid DNA. 20 fmol of pSarg (60 ng) or pPM5 (85 ng) were then added in 2 μl buffer containing 5 mM $MgCl_2$ and 150 mM KCl. Incubation was continued at 25°C for 5 min (10 min in the experiment of Figure 5) before adding the remaining components of the standard transcription reaction. From this point, the procedure was exactly as described for the standard reaction (see above).

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