Interaction of Yeast Transcription Factor IIIC with Dimeric Schizosaccharomyces pombe tRNA<sup>Ser</sup>-tRNA<sup>Met</sup> Genes\*  

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A unique tRNA<sup>Ser</sup>-tRNA<sup>Met</sup> tandem gene arrangement was characterized previously from Schizosaccharomyces pombe. Three alleles exist in which a tRNA<sup>Met</sup> gene is separated by 7 base pairs from an initiator tRNA<sup>Ser</sup> gene. Promotion of transcription occurs only within the tRNA<sup>Ser</sup> gene, yielding a dimeric precursor transcript. Using nuclease protection and gel retardation assays, we have analyzed how the Schizosaccharomyces cerevisiae RNA polymerase III transcription factor C (TFIIC) interacts with this dimeric gene template. The primary interaction site of TFIIC with the tRNA<sup>Ser</sup> gene is at the 3'-internal control region (ICR), which can be distinguished kinetically from its weaker interaction with the 5'-ICR of the gene. We examined a variety of point mutations and double mutations within the tRNA<sup>Ser</sup> gene which reduce transcription. We found that changes in highly conserved nucleotides within the ICRs reduce TFIIC binding up to 7-fold compared with the parent suppressor gene. The interaction of TFIIC with the tRNA<sup>Ser</sup> gene does not sterically prevent stable binding of TFIIC to the 3'-ICR of the tRNA<sup>Met</sup> gene. However, the affinity of binding of TFIIC to the dimeric template is 7-fold higher than to the tRNA<sup>Met</sup> gene, alone, demonstrating that the tRNA<sup>Met</sup> gene contains intrinsically weak promoter elements. This may contribute to the inability of the tRNA<sup>Met</sup> gene to independently direct transcription from its ICR elements.

Transcription of eucaryotic tRNA genes involves RNA polymerase III and at least two protein components designated as TFIIB and TFIIC.\* These genes contain two internal control regions (ICRs) where the 5'-ICR corresponds to the D-loop and the 3'-ICR corresponds to the T-loop in the resultant tRNA. These regions contain highly conserved nucleotide sequences that are necessary for tRNA function. Mutations in these have revealed that these nucleotides are also important in promoting transcription and TFIIC interactions (for reviews see Refs. 1 and 2).

The transcription process involves the formation of stable preinitiation complexes (3, 4). TFIIB, TFIIC, and the DNA template form a complex which is stable for many rounds of transcription initiation. The various protein-protein and protein-DNA interactions which specify this complex remain undefined. The ability of TFIIC to stably interact with tRNA genes has been demonstrated by studies involving template competition (4, 5), nuclease protection (6, 7), and gel retardation analysis of TFIIC-template complexes (8, 9). These studies show that the primary interaction site of TFIIC is within the 3'-ICR (8, 10, 11). The extent of interaction with the 5'-ICR appears to be template dependent and is affected by the distance separating the two ICRs (11, 12).

Genes transcribed by RNA polymerase III are generally transcribed as monocistronic units (1). In yeast, about 350 tRNA genes are dispersed within the genome, and only a few of these are clustered (13). In Saccharomyces cerevisiae there are two alleles containing a tRNA<sup>Thr</sup>-tRNA<sup>Asp</sup> gene pair (14), and in Schizosaccharomyces pombe these are three alleles containing a tRNA<sup>Ser</sup>-tRNA<sup>Met</sup> gene pair (15). These closely spaced gene pairs, separated by 10 or 7 base pairs, respectively, give rise to dimeric precursor transcripts. The three S. pombe tRNA<sup>Ser</sup>-tRNA<sup>Met</sup> gene pairs were identified previously by genetic analysis of tRNA-mediated nonsense suppression (19, 22). The sup<sup>3</sup>-e, sup<sup>9</sup>-e, and sup<sup>12</sup>-e loci of S. pombe encode tRNA<sup>Ser</sup> genes and initiator tRNA<sup>Met</sup> genes (15). These dimeric genes give rise to a transcript of approximately 190-nucleotides, and they differ by a single base at the tip of the extra arm of the serine tRNA and in the sequences flanking these two genes. Subsequent studies have characterized a variety of both in vivo-derived sup<sup>3</sup> and sup<sup>9</sup> revertant alleles (20, 21) as well as in vitro-derived point mutations within the sup<sup>3</sup>-e fragment (18). This has allowed for the identification of mutations affecting transcription and various RNA processing steps, including exclusion of the intron within the tRNA<sup>Ser</sup> transcript.

For the dimeric gene templates, transcription is directed by the promoter elements of the first (5') gene and independent transcription promotion of the second gene does not occur (16, 17). The reason for this unique tRNA gene arrangement and the resultant coordinated expression is unclear. Mutational analysis of the tRNA<sup>Ser</sup>-tRNA<sup>Met</sup> gene pair of S. pombe has revealed that even when the first gene is inactivated, the second gene is still incapable of promoting its own transcription (18). However, deletion of the tRNA<sup>Thr</sup> gene and spacer region from the tRNA<sup>Thr</sup>-tRNA<sup>Asp</sup> gene template of S. cerevisiae allows the tRNA<sup>Asp</sup> gene to be transcribed (17). This suggests that the inability of the tRNA<sup>Asp</sup> gene to direct transcription may be due, in part, to sequences within the tRNA<sup>Asp</sup> gene and the spacer region which flank the tRNA<sup>Met</sup> gene and inhibit transcription. By further defining how the transcription components interact with these dimeric genes, we may better understand the mechanism by which their

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\* The abbreviations used are: TF, transcription factor; ICR, internal control region; bp, base pair(s); Hepes, 4-(2-hydroxyethyl)-1-piperazinenuethanesulfonic acid.
In order to gain insight into the reason for the unusual dimeric gene arrangement and resultant transcription properties, we have further analyzed a set of transcription defective mutants in the sup3-e and sup9-e alleles. We have examined both qualitatively and quantitatively the interaction of S. cerevisiae TFIIIC with the parent and mutant gene templates. These results identified specific nucleotides within the tRNA\textsuperscript{Ser} gene which are necessary for TFIIIC interaction. Although the binding of TFIIIC to the tRNA\textsuperscript{Ser} gene does not sterically prevent TFIIIC binding to the adjacent tRNA\textsuperscript{Met} gene, the tRNA\textsuperscript{Ser} gene has a much reduced affinity for TFIIIC. Thus, one of the factors that may contribute to the inability of the tRNA\textsuperscript{Met} gene to be transcribed independently is its relatively weaker promoter elements which substantially reduce its ability to bind to TFIIIC.

**EXPERIMENTAL PROCEDURES**

Plasmid DNAs—S. pombe tRNA genes contained in a YRp17 plasmid include sup3-e (19) and sup9-e (20) which encode tRNA\textsuperscript{Ser} and tRNA\textsuperscript{Met} in a dimeric arrangement. The various mutant derivatives of sup3-e are shown in Fig. 1. pMet encodes the monomeric S. pombe tRNA\textsuperscript{Met} (23). pArg encodes a Drosophila tRNA\textsuperscript{Arg} gene contained in pBR322 (24).

Fragments derived from the plasmid DNAs used are diagrammed in Fig. 2. To quantify the amount of DNA used in the gel retardation assays, the method of Thomas and Farquhar (25) was employed using diaminobenzoic acid.

Preparation of TFIIIC—Strain 20B-12 (atrp 1 pep 4-3), a proteinase- and ribonuclease-deficient strain of S. cerevisiae, was used for the preparation of cell-free extracts. The soluble cell-free (S100) extracts were prepared as described by Klekamp and Weil (26). TFIIIC was partially purified as described by Johnson and Wilson (27) and contained no detectable TFIIID or RNA polymerase III activities. Preparations of TFIIIC were contained in 20 mM Hepes, pH 7.9, 2 mM EDTA, 0.2 mM dithiothreitol, and 100 mM NaCl at a concentration of approximately 10 mg/ml.

Nucleic Acid Protection Assays—DNase I protection assays were carried out using approximately 5 fmol (5,000-10,000 cpm) of 32P-end-labeled DNA fragments as described in Fig. 2. The TFIIIC fraction (10 µg) was incubated with the DNA fragment at 20 °C for the times indicated in 20 µl total volume of buffer A (20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 5 mM MgCl\textsubscript{2}, 100 mM NaCl, and 6% glycerol) containing 5 µg/ml bovine serum albumin. DNase I was added (2 µl of 50 ng, diluted from 1 mg/ml stock in buffer A containing 25 µg/ml bovine serum albumin and 0.5 mM CaCl\textsubscript{2}), and digestion was allowed to proceed at 20 °C for 1 min. The reaction was stopped by the addition of 40 µl of buffer B (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.2 mM sodium dodecyl sulfate, and 200 µg/ml sheared calf thymus DNA) and heating to 100 °C for 2 min. DNA fragments were extracted with phenol, precipitated with ethanol, and separated on 8% polyacrylamide gels containing 8 M urea. Gels were exposed to film with intensifier screens. To provide a ladder of reference DNA fragments, G-specific DNA sequencing reactions were carried out according to Maxam and Gilbert (28).

For the λ- exonuclease assays, the same conditions were employed for carrying out the TFIIIC-DNA binding reaction above. After incubation of the TFIIIC fraction with the labeled DNA probe for 20 min, the 20-µl reaction was diluted to 100 µl with buffer containing 20 mM Tris, pH 8.0, 3 mM NaCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 15% glycerol. λ-Exonuclease was added (5 µl of 1.5 units diluted from 0.6 units/µl stock) and incubated for either 2 or 10 min. The reaction was stopped by the addition of 80 µl of buffer B and held to 100 °C for 2 min. The DNA fragments were extracted and analyzed on 8% polyacrylamide gels as described for the DNase I protection assays.

DNA-binding Gel Retardation Assays—Each of the DNA binding reactions were performed in 20 µl of buffer C (20 mM Hepes, pH 7.9, 1 mM dithiothreitol, 4 mM MgCl\textsubscript{2}, 150 mM NaCl, 0.5 µg/ml bovine serum albumin, 0.05% Nonidet P-40, 5% glycerol) 1 µg of double-stranded poly(dI-dC), 0.5–1 ng of 32P-end-labeled DNA fragment, and unless otherwise indicated, 0.25–0.5 µg of the TFIIIC protein fraction.

To quantify the amount of complexed DNA, the DNA was visualized by autoradiography, and the retarded bands were excised from the gel and Cerenkov radiation measured. For each experiment, known amounts of the labeled probe were loaded on the gel in addition to the DNA-binding reactions. The amount of known DNA and the resultant Cerenkov radiation measured from the DNA bands was used to produce a standard curve. A combined plot of 11 standard curves was generated. Relative binding affinity of each mutant template was determined in triplicate with a competition binding experiment using 11 different concentrations of unlabeled DNA probe which spanned a 2000-fold range.
correcting for radioactive decay, the amount of bound DNA was determined from the slope and intercept of the combined standard curve. The resulting competition binding curves were fit to an equation for a single independent binding site using the program "ANALAB," an interactive nonlinear least squares regression analysis program for the IBM PC (29).

RESULTS

Analysis of TFIIC-tRNA Gene Template Interactions by Nuclease Protection Assays—We used DNase I protection assays to first determine where TFIIC interacts with the dimeric gene template and how mutations in either the 5′- or 3′-ICRs of the tRNA^Ser^ gene affect its binding. The fragments used for these and subsequent studies are diagrammed in Fig. 2. The DNA sequence of the sup3-e dimeric gene and the various point mutations examined are shown in Fig. 1. A partially purified S. cerevisiae TFIIC fraction was incubated for varying periods of time prior to digestion with DNase I with 32P-end-labeled fragments containing either sup9-e or suppressor-inactive mutants A19 or A53 (Fig. 3). The mutant templates were shown previously to have reduced transcription activities in vitro and in vivo (20). Analysis of the footprint pattern observed with the sup9-e fragment shows that extensive protection occurs within 1 min of incubation of the template with the factor in regions corresponding to the 3′-ICRs of the tRNA^Ser^ and tRNA^Met^ genes. Simultaneously, a DNase I hypersensitive region appears at the area immediately upstream of the protected 3′-ICR of the tRNA^Ser^ gene. This is most likely a result of a conformational change in the DNA induced by TFIIC binding. At the 5′-ICR of the tRNA^Ser^ gene, no protection is observed until after 20 min of factor incubation when the protection extends from the 5′-ICR through the 5′-flanking region of the gene. Although the extent of protection is less apparent than that observed in the 3′-ICR, it is reproducible. Incubating for longer times or increasing the amount of the TFIIC fraction did not increase the protection observed (data not shown). No protection in the 5′-region of the tRNA^Met^ gene was observed.

Comparison of the protection pattern of the mutant A19 to that of sup9-e, which changes a G to an A at position 19 in the 5′-ICR of sup9-e, reveals that 3′-ICR binding of TFIIC is unaltered. In the 5′-ICR, however, we have consistently observed less protection after 20 min of TFIIC-template incubation compared to the sup9-e gene. The DNase I footprint analysis of A53, which changes a G to an A at position 53 in the 3′-ICR of sup9-e showed that protection of the 3′-ICR of the tRNA^Ser^ gene is greatly reduced. Similar results were obtained when other mutant derivatives of the sup3-e template were analyzed (data not shown). The interaction of TFIIC with the tRNA^Met^ gene was unaltered by mutations in the tRNA^Ser^ gene. Even with the decrease in binding of TFIIC to the A53 template, we failed to observe any protection in the 5′-region of the tRNA^Met^ gene.

To further map the borders of the interaction of TFIIC with the tRNA^Ser^ gene, λ-exonuclease experiments were performed (Fig. 4). Using the same conditions employed for DNase I protection assays, we incubated labeled fragments containing the sup9-e, A19, and A53 templates with TFIIC for 20 min. λ-Exonuclease was then added and incubated for

![Fig. 3. DNase I footprint analysis of the sup9-e dimeric gene fragment with TFIIC. Reactions were carried out as described under “Experimental Procedures” in the absence (-) or presence (+) of TFIIC. Incubation times for the formation of TFIIC-DNA complexes are designated. Each reaction contained a HpaI/SauI fragment of sup9-e DNA or the A19 or A53 mutations 32P-labeled at the 3′-end. A schematic representation of the dimeric gene fragment relative to the DNase I-generated labeled fragments is shown on the right. The hatched boxes represent the ICRs of the tRNA^Ser^ and tRNA^Met^ genes.](image-url)

![Fig. 4. λ-Exonuclease protection analysis to map the borders of TFIIC interaction with sup9-e. λ-Exonuclease assays were performed as described under “Experimental Procedures” using the parent sup9-e fragment or fragments containing the A19 or A53 point mutations. After TFIIC-DNA complexes were formed, λ-exonuclease was added and digestion was allowed to proceed for 2 or 10 min as indicated. The nuclease-resistant DNA fragments generated are indicated by arrows and the numbers refer to the positions relative to the first base pair (+1) of the coding sequence of the tRNA^Ser^ gene (Fig. 1). The reactions were carried out in the presence (+) or absence (−) of the TFIIC fraction. A shows the analysis of the 3′-border of TFIIC interactions with the tRNA^Ser^ gene using a HpaI/SauI sup9-e fragment 32P-labeled at the 3′-end. B shows the analysis of the 3′-border of TFIIC interactions with the tRNA^Ser^ gene using a HpaI/AvaI sup9-e fragment 32P-labeled at the 5′-end.](image-url)
an additional 2 or 10 min. Analysis of the 5'-boundary of TFIIIC interactions with HpaI/SacI end-labeled sup9-e fragments is shown in Fig. 4A. When the sup9-e and A19 templates are preincubated with TFIIIC and subsequently digested with λ-exonuclease, a fragment was obtained consistent with digestion proceeding to position 67 within the tRNA^Ser^ gene. This position is 7 bp upstream of the 3'-ICR (Fig. 1).

In the control lanes where no factor was included, no comparable fragment of this size accumulated. This fragment was stable for 10 min under the digestion conditions. We did not observe larger fragments using either template even at shorter incubation times. Such fragments could correspond to the interaction of the factor with the 5'-portion of the tRNA^Ser^ gene. Therefore, under the conditions used, we did not observe interaction of TFIIIC with the 5'-ICR. The A53 template, which showed reduced affinity for TFIIIC by DNase I protection analysis, also showed less accumulation of the digestion-resistant band compared with that observed for sup9-e and A19.

The 3'-border of interaction of TFIIIC with the tRNA^Ser^ gene was determined by using a HpaI/AvaII fragment (Fig. 2). The fragment does not contain the 3'-ICR of the tRNA^Met^ gene and therefore allowed analysis of the binding of TFIIIC to the 3'-ICR of the tRNA^Ser^ gene. As shown in Fig. 4B, λ-exonuclease digestion for either sup9-e or A19 containing fragments preincubated with TFIIIC produces two discrete fragments. These correspond to positions 3 and 5 bp within the mature coding sequence of the tRNA^Met^ gene (Fig. 1). The A53 mutant gene showed less accumulation of these λ-exonuclease-generated fragments. These results are consistent with the DNase I protection assays which demonstrate that the primary interaction site of TFIIIC with the tRNA^Ser^ gene is a region of approximately 40 bp that includes the 3'-ICR. Within this region, a G to A change at position 53 reduces the ability of TFIIIC to bind stably. The interaction of TFIIIC with the 5'-region of the tRNA^Ser^ gene appears to be considerably less stable than it's interaction with the 3'-region.

**Analysis of TFIIIC-Template Interactions by Gel Retardation Assays**—The nuclease protection assays allowed us to qualitatively assess where TFIIIC interacts with the dimeric gene template and how this interaction is affected by point mutations within the tRNA^Ser^ gene. To further quantitate differences in TFIIIC interactions with this template and various mutant derivatives, we examined TFIIIC-tRNA gene complexes using a gel retardation assay. In this assay protein-DNA complexes are separated electrophoretically from unbound DNA. Thus, the amount of specific TFIIIC-DNA complexes can be quantified. This assay has been used previously to examine the effects of point mutations within a tRNA^Tyr^ gene on yeast TFIIIC binding by determining apparent equilibrium binding constants of these DNA templates (8).

To optimize conditions for examining specific TFIIIC-DNA complexes, each reaction contained a constant amount of a 32P-end-labeled 343-bp HindIII/AvaII fragment containing the sup3-e (Fig. 2) gene and a constant amount of poly(dI-dC) (to sequester nonspecific DNA binding proteins). We systematically varied the amount of the TFIIIC fraction in the reactions, and the reaction mixtures, after incubation for 45 min, were subjected to gel electrophoresis (Fig. 5A). With the addition of the TFIIIC-fraction (0.6–1.2 µg of protein), a discrete retarded band was observed, compared with the migration of free DNA observed in the reaction containing no TFIIIC. At higher concentrations of TFIIIC, larger aggregated complexes were formed. Using the appropriate amount of the TFIIIC fraction which produced discrete complexes, we next determined whether this complex represented a specific TFIIIC-tRNA gene interaction by performing competition experiments (Fig. 5B). The addition of increasing amounts of a plasmid containing a Drosophila tRNA^Arg^ gene (left lanes) or plasmid alone (right lanes) to each of the reactions revealed that the complex was dissociated only by competition with the tRNA gene. Thus, we conclude that this complex represents a specific TFIIIC-tRNA gene complex.

In order to quantitate the relative affinity of TFIIIC for the dimeric gene and the various mutant derivatives, we employed this gel retardation assay to determine the ability of each
template to compete for TFIIIC binding to a fragment containing the parent suppressor tRNA<sup>s<sub>er</sub></sup> gene. To first ensure that TFIIIC-DNA complex formation had reached equilibrium, a time course for incubation of the TFIIIC fraction with the DNA fragment prior to resolution of the complexes on polyacrylamide gels was performed. We found that a 45-min incubation period was the optimal time for equilibrium to be established (data not shown).

For consistency, we compared fragments of identical sizes and extragenic sequences (except for the mutations introduced). We, therefore, chose to analyze the variety of mutant and extragenic sequences (except for the mutations introduced in the dimeric gene fragment containing the tRNA<sup>s<sub>er</sub></sup> and tRNA<sup>m<sub>et</sub></sup> genes (Fig. 2) were added to the reactions so that the concentration range of amounts of a given unlabeled template HindIII/KpnI fragment was varied over 2000-fold and was present at concentrations between approximately 800-fold molar excess and 0.4-fold molar excess relative to the labeled fragment. The concentration of competitor DNAs within this range was insignificant compared with the large amount of poly(dI-dC) used in each reaction. We found 2 µg of poly(dI-dC) could be used in our assays without altering the amount of TFIIIC-DNA complexes formed (data not shown). For these studies, three independent sets of experiments were carried out. Each set consisted of the analysis of each competitor DNA using the same preparation of labeled DNA probe and TFIIIC preparation.

Fig. 6 (inset) shows a typical experiment in which there is a decreasing amount of bound labeled DNA as the concentration of competitor DNA is increased. After correction for radioactive decay, this data was analyzed by nonlinear least squares regression to determine the concentration of unlabeled competitor DNA required for 50% inhibition (IC<sub>50</sub>). The IC<sub>50</sub> was determined using the following equation.

\[
[C] = \frac{[ND] + [TD]}{1 + [UC]} IC_{50}
\]

where C is displaceable labeled DNA in complex (measured), ND is non-displaceable labeled DNA in complex (at high concentrations of unlabeled competitor DNA), determined by nonlinear fitting, TD is total labeled DNA in complex (at conditions where little or no unlabeled competitor DNA is added), determined by nonlinear fitting, UC is unlabeled competitor DNA, and IC<sub>50</sub> is concentration of unlabeled competitor DNA required for half-maximal inhibition of C binding to labeled DNA probe.

Table I gives the IC<sub>50</sub> values and the parameter standard error for the parent suppressor, mutant genes, and the tRNA<sup>s<sub>er</sub></sup> gene. All of the mutations introduced in the dimeric template were within the tRNA<sup>s<sub>er</sub></sup> gene and change highly conserved nucleotides within the ICRs (with the exception of G48) that decrease transcription of the template in vitro. For the mutant templates examined, there does not appear to be a correlation between which ICR is mutated and the magnitude of the decrease in TFIIIC binding. For the 5'-ICR mutations examined, only C14 showed significantly reduced TFIIIC binding that was 50% of the parent template. The most deleterious single point mutation is a C to G change at position 56 in the 3'-ICR. This mutant binds to TFIIIC 14% as well as the parent template. The double mutants, T18A55 and A19G56, showed substantial decreases in their ability to

**TABLE I**

Comparison of transcription, stable complex, and TFIIIC binding analysis of the tRNA<sup>s<sub>er</sub></sup>-tRNA<sup>m<sub>et</sub></sup> gene and mutant templates

| Mutation | Base change | Relative transcription efficiency<sup>a</sup> | Relative stable complex formation | Relative binding affinity of TFIIIC | IC<sub>50</sub> |
|----------|-------------|--------------------------------|---------------------------------|----------------------------------|----------------|
| sup3-e   | Parent      | 100                          | 100                             | 100                              | 0.60 ± 0.20    |
| C14      | A → C       | 78                           | 83                              | 50                               | 1.2 ± 0.49     |
| T18      | G → T       | 68                           | 95                              | 125                              | 0.48 ± 0.14    |
| A19      | G → A       | 45                           | 76                              | 81                               | 0.74 ± 0.26    |
| G48      | C → G       | 50                           | 63                              | 115                              | 0.52 ± 0.17    |
| A55      | T → A       | 75                           | 70                              | 82                               | 0.73 ± 0.22    |
| G56      | C → G       | 3                            | 5                               | 14                               | 4.2 ± 4.7      |
| T58      | A → T       | 30                           | 45                              | 83                               | 0.72 ± 0.29    |
| T18A55   | G → T, T → A | 55                          | 55                              | 21                               | 2.9 ± 1.95     |
| A19G56   | G → A, C → G | 1                           | 5                               | 24                               | 2.5 ± 1.19     |

<sup>a</sup> Data from Nichols et al. (18).
bind to TFIIIC compared with the single point mutants. As the relative affinity of TFIIIC for the templates decreases, however, the error in measuring TFIIIC binding increases. It is, therefore, difficult to assess whether the introduction of double mutations in both ICRs has more than an additive effect on TFIIIC binding compared with templates containing only one of the mutations. The ability of the tRNA$^{Ser}$ gene to compete for TFIIIC binding is significantly less than the dimeric gene template.

Table I also shows the relative abilities of the dimeric gene templates to be transcribed and to form stable transcription complexes. The stable complex formation assay measures the ability of TFIIIC and TFIIIB to interact stably with the tRNA gene template (3). Using these two assays and comparing the mutant gene templates, the trend is similar to that observed for their relative abilities to interact with TFIIIC. The G56 mutation caused the most dramatic reduction in reducing template activity and in the formation of stable transcription complexes. The double mutations in the tRNA$^{Ser}$ gene caused a much greater decrease in transcription and stable complex formation compared with single mutations in the gene.

**DISCUSSION**

Our examination of the interaction of TFIIIC with the dimeric gene template by nuclease protection assays revealed three distinct binding sites. Rapid binding of the protein occurs at the regions corresponding to the 3'-ICRs of both tRNA$^{Ser}$ and tRNA$^{Met}$ genes. Approximately 40 bp of DNA is protected in the 3'-half of the tRNA$^{Ser}$ gene extending into the 5'-region of the tRNA$^{Met}$ gene. Less rapid and less complete protection occurs in a region corresponding to the 5'-ICR of the tRNA$^{Ser}$ gene and extends into the 5'-flanking region. Nuclease protection analysis showed that TFIIIC did not interact with the 5'-ICR of the tRNA$^{Ser}$ gene either contained within the dimeric arrangement (Fig. 3) or separated from the tRNA$^{Ser}$ gene (data not shown).

Previous studies have shown that the binding of yeast TFIIIC to the 5'-ICR of tRNA genes is template-dependent. In some cases, complete protection through the entire gene is observed and factor binding to the 5'- and 3'-ICRs is kinetically indistinguishable (7). Some templates exhibit a less stable interaction of TFIIIC with the 5'-ICR. This interaction can be kinetically distinguished from its binding to the 3'-ICR and is more sensitive to ionic strength (10). The differences in 5'-ICR interactions displayed by different templates could be the result of both the "strength" of the promoter element as well as the distance separating the ICRs. The analysis of TFIIIC interaction with the yeast tRNA$^{Ser}$ gene revealed a pattern of protection similar to that observed with the tRNA$^{Ser}$ gene (10). Both of these genes contain intervening sequences which increase the separation between the two ICRs. In the case of the tRNA$^{Ser}$ gene, the stability of TFIIIC interactions with the 5'-ICR increased when the distance separating the ICRs was shortened (8). The presence of the intron in the tRNA$^{Ser}$ gene, therefore, probably destabilizes the interaction of TFIIIC with the 5'-ICR of the gene.

The binding of TFIIIC to the tRNA$^{Ser}$ gene does not appear to sterically prevent its binding to the tRNA$^{Met}$ gene. Nuclease protection in the 3'-region of the tRNA$^{Met}$ gene is conserved regardless of the interaction of TFIIIC with the tRNA$^{Ser}$ gene. The apparent inability of TFIIIC to interact stably with the 5'-ICR of the tRNA$^{Met}$ gene even when TFIIIC interaction with the tRNA$^{Ser}$ gene is decreased may be the result of a "weaker" consensus sequence in this region. In other eukaryotic tRNA genes, position +11 in the mature coding sequence encodes a pyrimidine that is base-paired to a purine in the D-}

stem of the resultant tRNA (1). The tRNA$^{Ser}$ gene, however, encodes a purine (G residue) at this position (Fig. 1).

Although the nuclease protection studies allowed us to examine where TFIIIC interacted with the dimeric gene template, these experiments were performed using excess TFIIIC to saturate the binding sites on the DNA. In order to further examine more quantitatively the relative ability of TFIIIC to bind to the dimeric gene template when mutations are introduced into the tRNA$^{Ser}$ gene, a gel retardation assay was used. Analysis of 5'-ICR mutations showed at most a 2-fold reduction in TFIIIC affinity for the dimeric gene template. The single mutation, G56, and double mutations, T18A55 and A19G56, exhibited the most pronounced decrease in TFIIIC binding. These mutations showed a 4- to 7-fold decrease in TFIIIC affinity.

Previous studies have used the gel retardation assay to examine the effects of tRNA$^{Ser}$ gene promoter mutations on yeast TFIIIC binding (8). In this case, point mutations in the 5'-ICR also caused at most a 2-fold reduction in the binding constant. Mutations in the 3'-ICR, however, had a more dramatic effect on TFIIIC binding. The A54 and C57 mutations reduced the binding constant 43- to 56-fold, respectively. The G56 mutation in the tRNA$^{Ser}$ gene exhibited a 370-fold reduction in the ability of TFIIIC to bind. The large difference observed in the effects of 5'-ICR mutations on TFIIIC binding for the tRNA$^{Ser}$ gene compared with our results with the tRNA$^{Met}$ gene may be a result of differences in our assays. For the tRNA$^{Ser}$ gene, the concentration of each mutant template varied in the assay to measure relative association constants, whereas our assay used varying concentrations of the dimeric mutant templates as competitors in the presence of a fixed concentration of the tRNA$^{Ser}$ gene to measure inhibition of TFIIIC binding to the tRNA$^{Ser}$ gene. It is also possible that the interaction of TFIIIC with the tRNA$^{Met}$ gene stabilizes the interaction of the protein bound to the adjacent tRNA$^{Ser}$ gene. Mutations in the 3'-ICR which would normally substantially reduce TFIIIC binding to this site may be compensated to some degree by a cooperative interaction of TFIIIC bound to the tRNA$^{Met}$ gene.

Analysis of the tRNA$^{Ser}$ gene-TFIIIC interaction showed it to be comparable to TFIIIC's interaction with the G56 mutation in the tRNA$^{Ser}$ gene. This result is further substantiated by examination of these templates using the stable complex formation assay. The G56 mutation contained within the dimeric gene fragment competes for the transcription components 5% as well as the parent dimeric gene template. These results demonstrate that the tRNA$^{Met}$ gene, contained within the dimeric gene arrangement or separated from the tRNA$^{Ser}$ gene, binds to TFIIIC with a much reduced affinity compared with the tRNA$^{Ser}$ gene. This results in a decreased ability of the tRNA$^{Ser}$ gene to form stable transcription complexes and therefore decreases the rate of transcription from this gene. These results may explain the unusual arrangement and transcription properties of these two genes. The coupling of the tRNA$^{Met}$ gene to the transcription of the tRNA$^{Ser}$ gene could provide means to increase the transcription rate of a normally poorly transcribed gene. If the cellular levels of TFIIIC are limiting compared with the number of RNA polymerase III genes, the relatively weaker promoter elements of the tRNA$^{Met}$ gene may not be bound by TFIIIC. The adjacent tRNA$^{Ser}$ gene with relatively stronger promoter elements would be bound by TFIIIC. Transcription from the tRNA$^{Ser}$ gene would occur producing both tRNA$^{Ser}$ and tRNA$^{Met}$ products. Baker et al. (8) have compared the interaction of yeast TFIIIC with S. cerevisiae tRNA$^{Ser}$ and tRNA$^{Met}$ genes and found these two genes to have specific
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binding constants which differed almost 10-fold (8). Further comparisons of the relative binding affinities for different TFIIIC-tRNA gene complexes may provide insight into how transcription of these genes is regulated to meet codon use.

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