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Comparison of tRNA Gene Transcription Complexes Formed In Vitro and in Nuclei

JON M. HUIBREGTSE, CLAIRE F. EVANS, AND DAVID R. ENGELKE*

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

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The nucleoprotein structure of single-copy tRNA genes in yeast nuclei was examined by DNase I footprinting and compared with that of complexes formed in vitro between the same genes and transcription factor C. Transcription factor C bound to both the 5′ and 3′ intragenic promoters of the $tRNA^{Leu}_{SUP53}$ gene in vitro, protecting approximately 30 base pairs at the 3′ promoter (B block) and 40 base pairs at the 5′ promoter (A block) and causing enhanced DNase I cleavages between the protected regions. Binding to the two sites was independent of the relative orientation of the two sites on the helix and was eliminated by a single point mutation in the 3′ promoter. The chromosomal $tRNA^{Leu}_{SUP53}$ and $tRNA^{Ser}_{UCG}$ genes showed a pattern of protection and enhanced cleavages similar to that observed in vitro, indicating that the stable complexes formed in vitro accurately reflect at least some aspects of the nucleoprotein structure of the genes in chromatin.

The transcription of eucaryotic tRNA genes is controlled by two highly conserved intragenic sequence elements located near the 5′ and 3′ ends of the genes, termed the A and B blocks, and by more variable sequences near the site of transcription initiation (9, 16, 18, 38, 40; reviewed in reference 39). The internal elements are also essential to tRNA processing and function, even in procaryotes, and may therefore have evolved into ubiquitous eucaryotic promoters as tRNA genes became independent transcription units. The upstream sequences have been shown to influence transcription in a species-specific manner (10, 12, 40) and have been postulated to impose differential regulation on classes of tRNA genes within an organism (14, 23, 29, 47). The precise mechanism by which these promoter elements direct RNA polymerase III initiation is not clear, but an early step in gene activation appears to be the association of one or more transcription factors with the A and B blocks.

Fractionation of cellular extracts has identified at least two components, transcription factor B (TFIIIB) and TFIIIC, that are required in addition to RNA polymerase III for tRNA transcription in vitro (17, 28, 37). TFIIIC contains an activity that binds to both the A and B regions in vitro (7, 8, 44) and remains bound through multiple rounds of gene transcription (3, 17, 34). Experiments with altered tRNA gene templates have shown that binding is primarily directed by the B block, although A block mutations lower the overall binding efficiency and drastically reduce promoter strength (2, 7, 31, 44). The function of TFIIIB is unclear, although it has been shown to be necessary for the formation of stable preinitiation complexes in some cases (3).

The spacing between the A and B block promoter regions varies from 30 to 80 base pairs in naturally occurring tRNA genes (9, 39, 41). The binding of a single TFIIIC complex to two sites separated by such a variable length of DNA would require considerable flexibility in the structure of the factor. It has been proposed instead that the DNA is contorted to allow the promoter elements to attain a fixed proximity (22, 42). A configuration where the DNA between the two sites is bent when TFIIIC is bound has been supported by electron micrographs of complexes formed in vitro (42) and could contribute to the observed nuclease hypersensitivity of tRNA genes in chromatin (11, 13).

The degree to which in vitro observations of transcription factor-DNA interactions reflect the physical state of tRNA genes in vivo has yet to be determined. To address this problem we have examined the nucleoprotein structure of single-copy chromosomal tRNA genes in yeast nuclei by high-resolution DNase I footprinting. The results demonstrate that stable complexes formed between TFIIIC and tRNA gene internal promoters in vitro represent valid models for events occurring on chromosomal tRNA genes.

MATERIALS AND METHODS

Yeast strains. All yeast strains used in these studies were transformants of *Saccharomyces cerevisiae* DC5 (*leu2-3 leu2-112 can1-11*; obtained from Cold Spring Harbor Laboratory). Yeast transformations were done by the lithium acetate procedure of Ito et al. (26) with linearized M13 double-stranded DNA containing a functional *LEU2* gene and an altered $tRNA^{Leu}_3$ gene as previously described (25). Cells were grown in YPD medium containing 2% glucose (Sigma Chemical Co.), 2% peptone, and 1% yeast extract (Difco Laboratories).

Plasmids. Recombinant M13 viruses containing the $tRNA^{Leu}_{SUP53}$ gene variants were obtained from A. Newman, M. Strobel, and J. Abelson (31, 45). For the in vitro transcription and footprinting studies, DNA fragments containing sequences from the *Xho*I site to an *Alu*I site within the *LEU2* coding region (31) (Fig. 1) were excised from the M13 polylinker region with *Eco*RI and *Hin*dIII and inserted into the same polylinker sites of plasmid pUC9. Plasmids were carried in *Escherichia coli* JM101 and purified by the method of Birnboim and Doly (5).

Chromatographic fractionation of transcription factors. Yeast nuclear extract was prepared from *S. cerevisiae* EJ101 by a modification of the method of Weiderrecht et al. (46), essentially as described previously (15). Yeasts were harvested in late logarithmic growth and washed with isotonic buffer (1.0 M sorbitol, 50 mM Tris [pH 7.9], 10 mM $MgCl_2$) containing 30 mM dithiothreitol (DTT). The yeasts were then digested with Zymolyase (33 ml of isotonic buffer containing 3 mM DTT per liter of cells and 0.15 mg of Zymolyase 60,000 [ICN Pharmaceuticals] per ml) for 60 min at 30°C, followed

* Corresponding author.

by lysis in 21 ml of hypotonic buffer (15 mM KCl, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0), 5 mM $MgCl_2$, 0.1 mM EDTA, and 3 mM DTT) per liter of cells. Crude nuclei were harvested by centrifugation in a Sorvall GSA rotor at 10,000 rpm for 20 min at 4°C and extracted in hypotonic buffer containing 0.8 M $(NH_4)_2SO_4$. After centrifugation at 34,000 rpm for 60 min in a Beckman 35 Ti rotor, the solubilized proteins were concentrated by precipitation in saturated $(NH_4)_2SO_4$ and suspended in one-half the nuclear pellet volume of 10% glycerol–20 mM HEPES (pH 8.0)–0.1 mM EDTA–1 mM DTT.

The following chromatography procedures were all performed at 4°C. The extract from 18 liters of yeast was loaded onto a 70-ml Bio-Rex 70 column (Bio-Rad Laboratories) in 0.32 HgKMED (20 mM HEPES [pH 7.9], 10% glycerol, 0.32 M KCl, 8 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT). After washes with several column volumes of 0.32 HgKMED, the RNA polymerase III, TFIIIB, and TFIIIC were eluted together in the same buffer containing 1.0 M KCl. These fractions were pooled, and the protein was precipitated by adding 0.45 g of ammonium sulfate per ml. The protein precipitate was suspended in 0.07 HgAED (20 mM HEPES [pH 7.9], 10% glycerol, 0.07 M ammonium sulfate, 0.1 mM EDTA, 2 mM DTT) and applied to a 35-ml DEAE-cellulose column (DE52; Whatman Ltd.). Under these conditions, TFIIIB flowed through the DE52 column, whereas TFIIIC and RNA polymerase III were retained. TFIIIC and RNA polymerase III were eluted with 0.2 HgAED, the fractions were pooled, and the salt was raised to 0.22 M ammonium sulfate. The pool was loaded onto a 10-ml DEAE-Sephadex column (A25; Pharmacia Fine Chemicals) equilibrated with 0.22 HgAED. TFIIIC flowed through this column, whereas RNA polymerase III bound. RNA polymerase III activity was eluted with 0.5 M ammonium sulfate and was assayed for catalytic activity with poly(dA-dT) template (36). The chromatographic fractions required in addition to RNA polymerase III for specific tRNA transcription were named by the convention of Segall et al. (37) on the basis of their elution profiles from DEAE ion exchangers (27) and on the basis of the binding of a component of the TFIIIC fraction to the intragenic promoters of a tRNA gene (17, 37). In transcription and footprinting assays 2-μl samples of the transcription component fractions were used.

In vitro transcription. Reactions were carried out for 20 min at 30°C in a total volume of 20 μl containing 0.2 mM each ATP, GTP, and CTP, 0.02 mM UTP, 0.06 μM [α-^{32}P]UTP (812 Ci/mmol), 140 mM KCl, 8 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.2 μg of plasmid containing the $tRNA^{Leu}_{SUP53}$ gene, and 2 μl of yeast nuclear extract or transcription factor fractions. The transcription reaction was stopped by adding 4 μl of stop mix containing 2% sodium dodecyl sulfate–0.1 M EDTA–1 mg of proteinase K (Beckman Instruments, Inc.) per ml–1 mg of tRNA per ml and incubating at 48°C for 45 min. The RNA was ethanol precipitated, suspended in 4 μl of formamide containing 0.1% xylene cyanol, and electrophoresed in a 10% polyacrylamide–8 M urea gel (35). Radiolabeled transcripts were detected by exposure to Kodak XAR5 film with a Du Pont Cronex Lightning-Plus intensifying screen for 12 h at −70°C.

In vitro footprinting. Supercoiled plasmids containing the $tRNA^{Leu}_{SUP53}$ gene or gene variants were used in primer extension footprinting assays. The supercoiled DNAs from large-scale plasmid preparations were excised and purified from low-melting-point agarose gels (Bethesda Research Laboratories, Inc.). The DNA was confirmed to be supercoiled by differential electrophoretic mobility in agarose gels before and after treatment with topoisomerase I (Promega Biotec Co.).

The method employed to detect binding of proteins to DNA was similar to the indirect footprinting technique developed by Gralla (20). Ten nanograms of supercoiled plasmid containing a tRNA gene and 0.5 μg of carrier DNA (pUC9 plasmid DNA) were incubated for 20 min at room temperature with or without 2 μl of transcription factor fraction in a final volume of 20 μl, containing 4 mM HEPES (pH 7.9), 45 mM ammonium sulfate, 6 mM $MgCl_2$, 2 mM DTT, 0.02 mM EDTA, and 2% glycerol. The preincubation with or without TFIIIC did not result in a noticeable loss of DNA superhelicity as judged by agarose gel mobility. Two microliters of 10-μg/ml DNase I (Worthington Diagnostics) was added, and the reaction was allowed to continue for 30 s at room temperature. The reaction was quenched by the addition of 4 μl of stop mix (2% sodium dodecyl sulfate, 0.1 M EDTA, 1 mg of proteinase K [Beckman] per ml). The reactions were incubated for 30 min at 50°C, followed by ethanol precipitation of the DNA. The DNA pellet was washed with 75% ethanol, dried, and suspended in 12 μl of water plus 1 μl of ^{32}P-labeled oligodeoxynucleotide (about 50,000 dpm; specific radioactivity, 5,000 Ci/mmol). The DNA was denatured at 95°C for 5 min and then placed at 50°C. Two microliters of 10× avian myoblastosis virus (AMV) reverse transcriptase buffer (0.5 M Tris [pH 8.3], 0.4 M KCl, 60 mM $MgCl_2$, 10 mM DTT) was added, and the mixture was incubated at 50°C for 20 min. Four microliters of 5× dNTP mix (2.5 mM each dATP, dTTP, dCTP, dGTP) and 1 μl (0.8 U) of AMV reverse transcriptase (Life Sciences, Inc.) were added, and the reactions were incubated for an additional 25 min at 50°C. The reactions were quenched with stop mix, incubated at 50°C for 30 min, ethanol precipitated, washed with 75% ethanol, and dried. Pellets were suspended in 4 μl of 95% deionized formamide–1 mM EDTA–10 mM NaOH–0.1% xylene cyanol, heated at 95°C for 2 min, and loaded onto 8% (0.15-mm by 40-cm) sequencing gels (35) containing 8 M urea. Radiolabeled primer extension products were detected by exposure to Kodak XAR5 film with a Du Pont Cronex Lightning-Plus intensifying screen for 16 to 48 h at −70°C. Size markers were provided by concomitant electrophoresis of dideoxynucleotide chain termination sequencing reactions with a plasmid containing the gene of interest as a template. Protection from and enhancement of DNase I cleavage was judged by visual comparison of cleavages with their nearest neighbors and with the overall degree of digestion in repeated experiments.

Genomic footprinting. Cultures of the DC5 transformants (100 ml) were grown in YPD medium to an optical density at 600 nm of 2.5. The cells were harvested, washed in 15 ml of 40 mM EDTA–90 mM 2-mercaptoethanol, suspended in 7 ml of 1 M sorbitol–1 mM EDTA–3 mM DTT–2 mg of Zymolyase 20,000 (ICN) per ml, and incubated at 30°C for 25 min with occasional swirling. Spheroplasts were collected by centrifugation at 3,000 × *g* for 5 min, suspended in 1.2 ml of digestion buffer (50 mM Tris [pH 7.4], 75 mM KCl, 6 mM $MgCl_2$, 0.5 mM $CaCl_2$, 3 mM DTT), and treated with 15 strokes of a loose pestle in a 7-ml Dounce homogenizer. Samples of cell lysate (0.3 ml) were immediately removed to a 1.5-ml microcentrifuge tube containing 30 μl of digestion buffer with various amounts of DNase I (0 to 0.8 mg/ml). DNase I digestions proceeded for 5 min at room temperature. Reactions were quenched by adding 0.33 ml of a solution containing 2% sodium dodecyl sulfate, 1 M NaCl, 50 mM EDTA, 50 mM Tris (pH 7.4), and 0.2 mg of proteinase

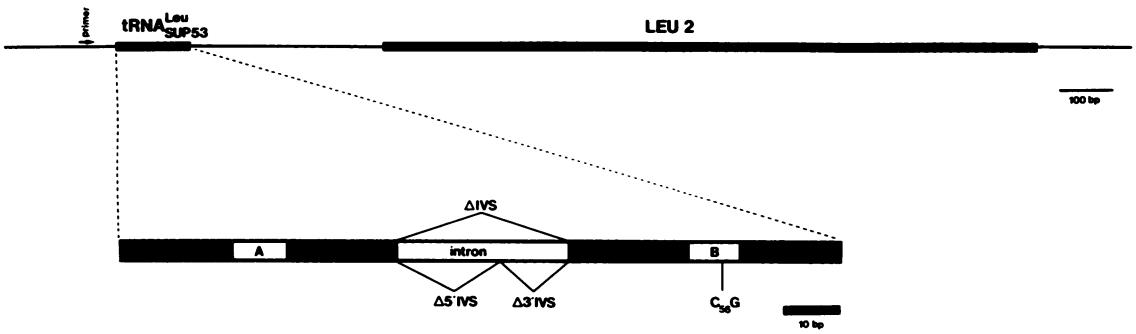

FIG. 1. Structure of the tRNA$^{Leu}_{SUP53}$ and *LEU2* genes. The DNA regions corresponding to the primary transcripts of the genes are indicated by the darkened bars (top). The small arrow (primer) indicates the region to which the oligonucleotide primer used to probe the tRNA$^{Leu}_{SUP53}$ gene hybridizes. The enlargement of the tRNA gene indicates the relative positions of the A and B block intragenic promoters and intron as well as the positions of the gene mutations used in this study. The $C_{56}G$ mutation is a C-to-G transition within the B block (31). The Δ5'IVS, Δ3'IVS, and ΔIVS mutations delete 13, 19, or 32 base pairs (bp), respectively, from the intron sequence (45).

K per ml. Reactions were placed at 50°C for 30 min and then extracted twice with an equal volume of phenol-chloroform (1:1). The nucleic acid was precipitated with an equal volume of isopropanol and suspended in 150 μl of TE (5 mM Tris [pH 7.9], 0.1 mM EDTA), containing 50 μg of RNase A per ml and left for 12 to 18 h at 37°C. The DNA was isopropanol precipitated again, suspended in TE, ethanol precipitated (2.5 volumes), and suspended in 50 μl of TE containing 20 μg of RNase A per ml. Each DNase I reaction yields enough DNA to perform 10 primer extension reactions (20 to 25 μg).

The DNase I cleavage sites of nuclear DNA were assayed by primer extension of a uniquely annealed ^{32}P-labeled oligonucleotide, as described above for footprinting on plasmids, with the following differences. Yeast genomic DNA (2 to 3 μg) was substituted for plasmid DNA, and 11 U of reverse transcriptase was used per reaction. The oligonucleotide (16-mer) used to probe the tRNA$^{Leu}_{SUP53}$ gene hybridized to the transcribed strand at positions −60 to −45 relative to the transcription initiation site (1, 25, 31). The oligonucleotide (19-mer) used to probe the tRNA$^{Ser}_{UCG}$ gene hybridized to the transcribed strand at positions −70 to −52 relative to the mature 5' end of the tRNA (32). When using the tRNA$^{Ser}_{UCG}$ oligonucleotide it was necessary to do the primer extension reactions at 52°C to ensure unique priming. Genomic sequencing reactions were done as described earlier (25).

DNase I digestions of deproteinized genomic DNA were done by combining 2 to 3 μg of the non-DNase I-digested nuclear DNA with 15 μl of digestion buffer and adding 2 μl of 0.9-μg/ml DNase I. The digestion was quenched after 30 s with 4 μl of 0.1 M EDTA. The DNA was ethanol precipitated and suspended in water plus ^{32}P-labeled oligonucleotide, and the DNase I cleavage sites were assayed by primer extension. DNase I concentrations in both nuclear digestions and naked DNA digestions were chosen to maximize the signal across the gene sequence. The pattern of cleavages remains the same until the signal disappears entirely through overdigestion, implying that it does not arise from a subset of genes in the cell population.

RESULTS

Chromosomal location and mutations of tRNA$^{Leu}_{SUP53}$ gene. The tRNA$^{Leu}_{SUP53}$ gene from chromosome III of *S. cerevisiae* was used for a majority of these experiments because the gene has been shown to be expressed in vivo (45) and because the proximity of the gene to *LEU2* (β-isopropylmalate dehydrogenase gene) facilitated the insertion of site-directed mutations of the tRNA$^{Leu}_{SUP53}$ gene at the correct chromosomal locus by gene replacement (25). The structure of the locus is shown in Fig. 1 and has been described previously (1). The mutations used in this study and their positions within the tRNA$^{Leu}_{SUP53}$ gene are also shown. The $C_{56}G$ mutation within the B block changes the C at tRNA position 56 to a G (standard tRNA nomenclature [25, 31]). This mutation eliminates all detectable TFIIIC binding in vitro and diminishes transcription at least 20-fold (31). The Δ3'IVS, Δ5'IVS, and ΔIVS mutations eliminate 13, 19, and 32 base pairs, respectively, from the intervening sequence. None of these deletion mutations noticeably affects the efficiency of in vitro transcription (45). As described elsewhere (25), the uptake of the tRNA$^{Leu}_{SUP53}$ gene or gene variants by gene replacement was directed by concomitant uptake of the *LEU2* gene into a *leu2-3112 S. cerevisiae* strain. The lack of any secondary mutations was confirmed by oligonucleotide primer extension sequencing with total yeast genomic DNA from the isolated transformants (25). These strain constructs allowed us to directly compare the binding of transcription factors to the tRNA$^{Leu}_{SUP53}$ gene and gene variants in vitro to nucleoprotein complexes on the same genes at the native chromosomal position.

TFIIIC binds to A and B blocks in vitro. The components of a *S. cerevisiae* extract necessary for tRNA gene transcription in vitro were separated by ion-exchange chromatography into three fractions containing RNA polymerase III, TFIIIB, and TFIIIC. To establish that the individual activities were free of detectable cross-contamination, transcription reactions were performed with plasmid DNA containing the tRNA$^{Leu}_{SUP53}$ gene as a template. Results with either crude extract or combinations of transcription factor fractions are shown in Fig. 2A. The two RNA species synthesized by the extract represent the primary transcript and the end-processed (5' and 3') tRNA (45). No factor alone (RNA polymerase III, TFIIIC, TFIIIB) or combination of two factors (RNA polymerase III plus TFIIIC, RNA polymerase III plus TFIIIB, or TFIIIB plus TFIIIC) was sufficient to reconstitute specific transcription, although RNA polymerase III synthesized high-molecular-weight RNA in the presence of either of the factor fractions. When all three fractions were combined, selective tRNA gene transcription was restored. Only the precursor tRNA species was seen in the reconstituted transcription reaction, because the 5' and 3' processing activities were removed during the chromatographic separation.

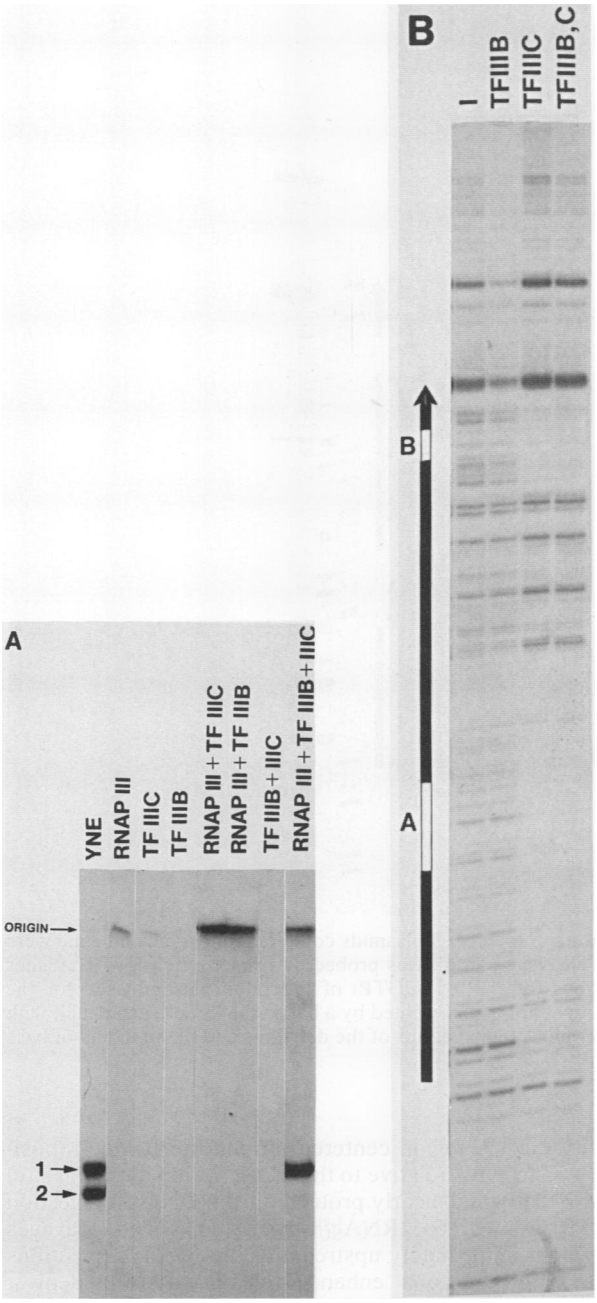

FIG. 2. In vitro transcription and DNase I footprinting of the $tRNA^{Leu}_{SUP53}$ gene with separated transcription factors. (A) Transcription reactions were performed as described in Materials and Methods with either crude yeast nuclear extract (YNE) or combinations of RNA polymerase III and transcription factor fractions. The RNA species labeled 1 and 2 represent the primary gene transcript and 5′ and 3′ end-processed transcript, respectively (15, 31, 45). (B) Supercoiled plasmids containing the $tRNA_{SUP53}{}^{Leu}$ gene were incubated without (−) or with the indicated transcription factor fractions and treated with DNase I, and the cleavage patterns were probed by primer extension as described in Materials and Methods. The region encoding the primary transcript is represented by the bar to the left of the panel, with the A and B block regions indicated.

A DNase I oligonucleotide primer extension footprinting technique was used to detect stable binding of transcription factors to supercoiled plasmids containing the $tRNA^{Leu}_{SUP53}$ gene. In the presence of enough TFIIIB to activate at least 25 times more template, TFIIIB does not bind stably to the $tRNA^{Leu}_{SUP53}$ gene (Fig. 2B). TFIIIC alone protects regions spanning both the A and B blocks (see Fig. 4), and the DNase I protection in the presence of both TFIIIB and TFIIIC is identical to that of TFIIIC alone. The same result was obtained when excess RNA polymerase III was present in addition to TFIIIB and TFIIIC (data not shown). Thus, although TFIIIB has been shown to stabilize TFIIIC binding in some cases (3), only TFIIIC appears to make direct, stable contacts with the internal promoter regions.

Effects of $tRNA^{Leu}_{SUP53}$ mutations on TFIIIC binding in vitro. Figure 3 shows the DNase I protection pattern due to TFIIIC binding to supercoiled plasmids containing the various altered $tRNA^{Leu}_{SUP53}$ genes. These data are summarized schematically in Fig. 4. DNA sequencing lanes across the region (Fig. 3, lanes C and G) provide position markers and internal controls for unique hybridization of the oligonucleotide primer. For each tRNA gene DNase I digestions are shown without (−) and with (+) TFIIIC. As already shown, TFIIIC protects regions spanning both the A and B blocks of the $tRNA^{Leu}_{SUP53}$ gene (Fig. 3, SUP53). The DNase I protection of the full-length and internally deleted SUP53 genes (SUP53, Δ3′IVS, Δ5′IVS, and ΔIVS) over the A block region is at positions +2 to +4, +9 to +38, and +43 to +48. Protection of the B block regions is over positions +93 to +122. The protected regions remain over the same sequence with increasing size of deletion, implying that interactions at both the A and B regions are sequence specific. The $C_{56}G$ mutation in the B block region eliminates all stable TFIIIC binding (Fig. 3, $C_{56}G$), in agreement with previous results (31). In addition to DNase I protection, enhanced DNase I cleavages are seen between the A and B block regions and near the 5′ border of the A block protection of the full-length and internally deleted SUP53 genes. The set of enhanced cleavages immediately upstream from the B block are at the same position on each gene (positions +86, +88, and +89). The enhancements between the A and B blocks are variable depending on the intron sequence. The enhancements in the SUP53 gene are separated by 10, 13, and 10 base pairs. The rightmost spacing shifts from 10 to 12 base pairs within the same sequence in Δ5′IVS. This may reflect only a shift in the cleavage preference controlled by surrounding DNA sequence or protein contacts, but could also signal a shift in the position of a helical distortion or DNA bend between the two binding sites (see Discussion). The Δ3′IVS gene has three sets of enhanced cleavages separated by 10 base pairs each. Each is at the same position with respect to nucleotide sequence as on the full-length gene. An enhanced cleavage at position +7 is seen on the full-length and deleted SUP53 genes but not on the $C_{56}G$ gene.

DNase I footprinting of chromosomal tRNA genes. To examine the nucleoprotein complexes assembled on chromosomal tRNA genes the primer extension DNase I footprinting technique was applied to yeast nuclei. Figure 5A shows genomic footprinting data for the $tRNA^{Leu}_{SUP53}$ (SUP53) and the $tRNA^{Leu}_{SUP53,C56G}$ ($C_{56}G$) genes. The DNase I cleavage pattern of deproteinized genomic DNA is shown in lane 1 in each case, with lane 2 being the DNase I cleavage pattern of nuclear DNA. Lane C is a genomic sequencing lane, which provides a control for unique hybridization of the oligonucleotide. The chromosomal SUP53 gene is only partially protected at the B block (positions +92 to +123), although

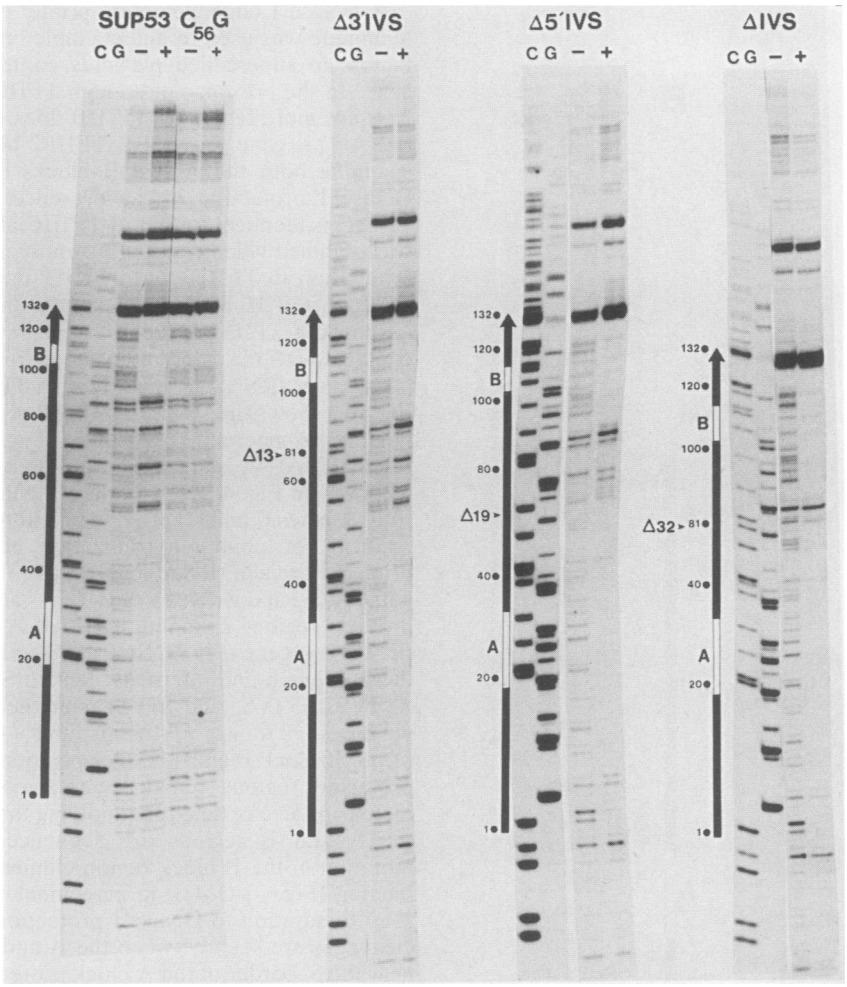

FIG. 3. In vitro DNase I footprinting of the $tRNA^{Leu}_{SUP53}$ gene and gene variants. Supercoiled plasmids containing the indicated gene were incubated without (−) or with (+) TFIIIC and treated with DNase I, and the cleavage pattern was probed by primer extension. The lanes labeled C and G are dideoxynucleotide chain-termination sequencing reactions (ddCTP or ddGTP) of the same plasmid used for the footprinting reactions (not shown for $C_{56}G$). The region encoding the primary transcript is represented by a bar to the left of each panel, with nucleotide positions indicated at 20-base-pair intervals. The A and B blocks as well as the positions of the deletions and the numbers of base pairs deleted are indicated.

the enhanced cleavages seen in the vicinity of the intron in plasmid footprinting are routinely present. Very little protection is evident in the region of the A block, although DNase I cleavages at positions +2 to +38 and +43 to +48 are reproducibly underrepresented. These are the same regions protected in vitro (Fig. 4), although the protection is more uniform at positions +5 to +8 and the enhanced cleavage seen in vitro at position +7 is absent. The $C_{56}G$ mutation, as in vitro, shows no A or B block protection. Slight enhancements in DNase I cleavage relative to that of the purified $C_{56}G$ genomic DNA are sometimes seen between the A and B blocks at positions +45 to +52, +60, and +63 to +66. The cause of this is unknown, but it may be an altered chromatin structure in the region, perhaps characteristic of a transcriptionally inactive tRNA gene.

To provide an internal control for the nuclear footprinting reactions, a second tRNA gene was probed with different samples of the same (strain DC5) SUP53 and $C_{56}G$ genomic DNAs. The single-copy $tRNA^{Ser}_{UCG}$ gene was chosen because it has been shown in vitro to have a particularly high affinity for TFIIIC (2) and because its expression is essential to viability (32). A region centered around the B block (positions +75 to +108 relative to the transcription initiation site) is consistently and clearly protected from DNase I digestion (Fig. 5B). As with the $tRNA^{Leu}_{SUP53}$ gene, enhanced cleavages are evident immediately upstream of the B block (positions +71 to +74; additional enhanced cleavages are evident at positions +38 and +48). In the experiment shown, the region around the A block is protected at positions +8 to +37 and +40 to +45, although the degree to which this region is protected relative to the B block varies between experiments.

The genomic footprinting data for the $tRNA^{Leu}_{SUP53}$ intervening sequence deletion genes are shown in Fig. 6. In contrast to the $tRNA^{Leu}_{SUP53}$ gene, both the A and B block protection and the enhanced DNase I cleavages are quite distinct and the same as observed for TFIIIC-plasmid DNA complexes, with the exception that protection is complete from positions −1 to +8. In addition, weak DNase I protection upstream of these genes from positions −5 to −13, as well as an enhanced cleavage at −14, are reproducibly seen. This effect is evident to a lesser degree on the full-length chromosomal SUP53 gene (Fig. 5A). The upstream protection and en-

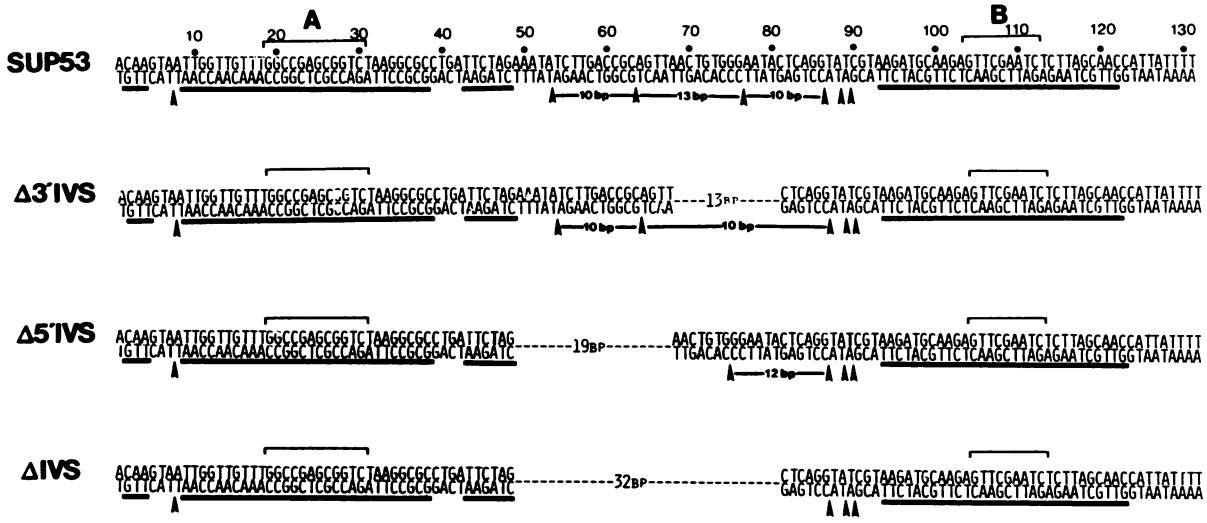

FIG. 4. Enhanced DNase I cleavages and protected regions in the $tRNA^{Leu}_{SUP53}$ gene and deletion variants due to TFIIIC binding in vitro. The DNA sequence encoding the primary transcript of each gene is shown with the A and B block internal promoters aligned. Nucleotide positions at 10-base-pair intervals are indicated above the SUP53 sequence. Dashed regions represent deleted sequences. The regions of the transcribed (lower) strand that are protected from DNase I by TFIIIC binding are indicated by a solid line. In regions where no cleavages exist in the naked DNA controls, positions between protected cleavages have been assigned as protected, whereas positions between a protected cleavage and a nonprotected cleavage have been assigned as unprotected. Enhanced DNase I cleavages are indicated by arrowheads with the distance in base pairs between adjacent enhanced cleavages indicated. Assignments are made cumulatively from the inspection of at least 10 experiments for each gene.

hanced cleavage are not seen with in vitro TFIIIC footprinting, nor are they seen on the chromosomal $C_{56}G$ variant.

DISCUSSION

The nucleoprotein structure of various altered chromosomal $tRNA^{Leu}_{SUP53}$ genes strongly resembles complexes formed in vitro on the same genes with transcription factor TFIIIC. This is particularly true for the gene variants with deletions of 13, 19, and 32 base pairs from the intron between the two TFIIIC binding sites, where both the A and B block promoter regions are strongly protected from DNase I. In contrast, promoter regions of the full-length gene, although they show patterns of protection and enhanced cleavages similar to the in vitro complexes, are less protected and therefore by implication less occupied by TFIIIC. Baker and Hall have shown that the optimum distance between the $tRNA^{Leu}_3$ A and B blocks (from G_{19} to C_{56}) is 38 to 57 base pairs in template competition experiments (3). This distance is 78, 65, 59, and 46 base pairs for the SUP53, Δ3'IVS, Δ5'IVS, and ΔIVS gene variants, respectively. As Baker and Hall pointed out (3), conditions of limiting TFIIIC in the nucleoplasm would be expected to favor occupation of the shorter genes with higher affinities for the factor.

The A block protection on the chromosomal SUP53 and $tRNA^{Ser}_{UCG}$ genes, in contrast to that of the internally deleted SUP53 genes, is consistently less complete than at the B block. This difference does not correspond to the relative affinities of these genes for TFIIIC, since the $tRNA^{Ser}_{UCG}$ gene has been shown to have a particularly high affinity for the factor (2, 3). This leads us to speculate that the degree of A block binding in vivo may reflect a particular transcriptional state of the gene. Some studies have shown TFIIIC binding only to the B block region in vitro (31) and A block associations have been shown to be selectively disrupted by low temperature, competition with single-stranded DNA (43), or limited proteolysis (30). If TFIIIC A block associations are more transient it is possible that they are regulated by factors not reproduced in vitro, such as the surrounding chromatin structure or competition with uncharacterized components of the nucleoplasm. It is also possible that the portion of TFIIIC that makes contacts with the A block can be entirely detached in vivo (30) and that our purification scheme has selected for a form which binds to both the A and B blocks.

Our in vitro and genomic footprinting data on a limited set of internally deleted SUP53 genes suggest that the relative helical orientation of the A and B blocks may not be critical to TFIIIC binding. This result is in agreement with binding studies with more extensive deletion and insertion mutations (R. Baker, personal communication) and with previous observations that the transcriptional efficiencies of these genes are similar (45). The same sequences over the A and B blocks in Δ3'IVS and Δ5'IVS, for example, are protected in vitro and on the chromosome even though the spacing between the two promoter regions differs by 6 base pairs, or one half turn of the helix. This result implies considerable flexibility in the binding of TFIIIC to the promoter sequences and is in marked contrast with the dependence of TFIIIA complex formation on the helical orientation of the 5S rRNA gene internal promoter elements (6, 33). Such flexibility could be inherent in the TFIIIC structure but might also be contributed by a distortion or bend in the DNA helix induced during binding. A bend has been observed in electron micrographs of the complex (42) and would explain how the two binding sites could be brought into fixed proximity even though in naturally occurring yeast tRNA genes the distance between the sites can differ by over 50 base pairs. Since inspection of available yeast nuclear tRNA gene sequences (41) does not reveal any obvious 10- or 11-base-pair repeat spacing motif between the A and B

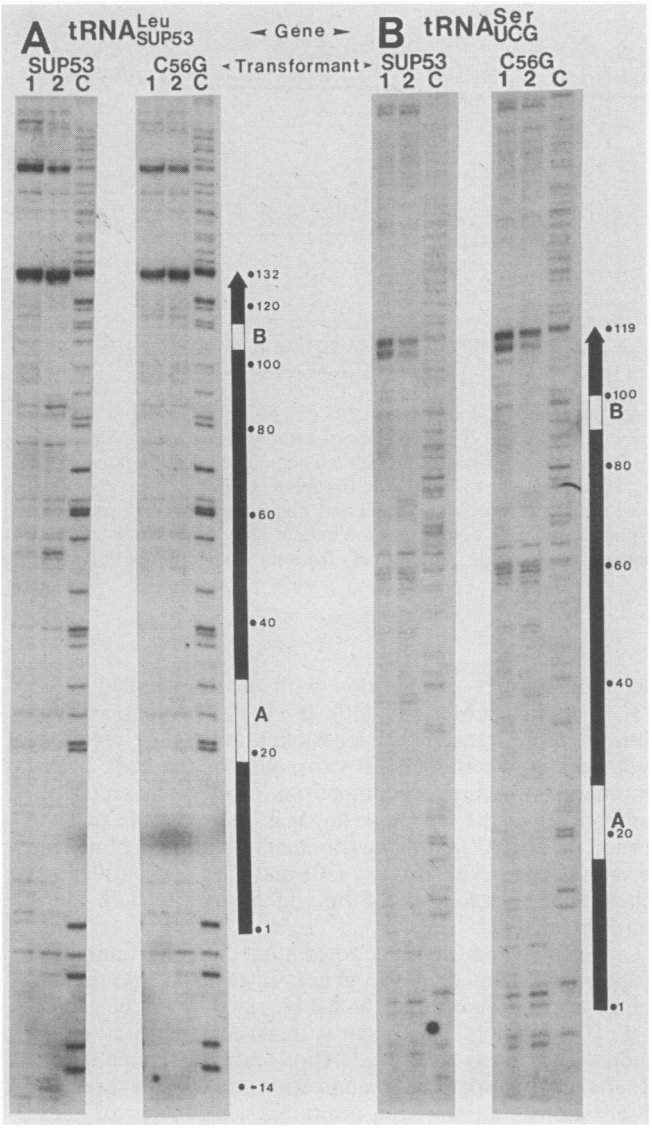

FIG. 5. DNase I footprinting of chromosomal tRNA$^{Leu}_{SUP53}$ and tRNA$^{Ser}_{UCG}$ genes. Exponentially growing cells transformed with either the SUP53 or $C_{56}G$ gene variants were converted to spheroplasts and gently lysed as described in Materials and Methods. Samples of cell lysate were immediately incubated for 5 min either without or with DNase I at a final concentration of 70 μg/ml, and the DNA was purified. Different samples of DNA from each reaction were probed with oligonucleotides that hybridized upstream of either the tRNA$^{Leu}_{SUP53}$ gene (A) or the tRNA$^{Ser}_{UCG}$ gene (B). Lanes: 1, in vitro DNase I digestions of deproteinized genomic DNA; 2, digestion pattern of genomic DNA in nuclei; C, dideoxy C sequencing reaction with undigested genomic DNA (25). The bars to the right of the panels represent the primary transcripts of the tRNA$^{Leu}_{SUP53}$ gene (15) and the tRNA$^{Ser}_{UCG}$ gene (32). Nucleotide positions are indicated at 20-base-pair intervals relative to the transcription initiation site.

blocks, this complex might also establish a constant spatial orientation by overwinding or underwinding the helix between the two sites.

It has been shown that when lambda repressor binds to two sites separated by six turns of the DNA helix, the intervening region adopts a bent conformation (21). DNase I cleavage enhancements in the intervening regions are spaced by about 10.5 base pairs on each strand (24), presumably due to preferential cleavage on the outer surface of the protein-DNA complex. In the case of the SUP53 gene, the DNase I cleavages enhanced by TFIIIC binding are spaced by 10, 13, and 10 base pairs (from left to right; Fig. 4). The 13-base-pair center spacing does not in itself signify a localized helical distortion, but it is interesting to note that the rightmost spacing shifts from 10 to 12 base pairs over unaltered sequences in the Δ5′IVS gene. If the 13-base-pair spacing on the SUP53 gene is due to a TFIIIC-induced helical distortion midway between the A and B blocks, that distortion would be expected to shift with the midpoint in the Δ5′IVS gene. The presence of only 10-base-pair spacings between enhanced cleavages in the Δ3′IVS gene could be taken to mean that the A and B blocks are in nearly the optimal helical orientation on this gene and the intervening DNA does not require a rotational correction. Additional physical studies of the complexes are currently under way to investigate the possibility of both bending and unwinding of the helix.

Although most of the upstream and downstream regions of the genes were not amenable to visualization by the probes used in these experiments, our preliminary observations of cleavage alterations immediately upstream of the tRNA^{Leu} gene variants make it clear that the structure of the surrounding chromatin warrants further investigation. The DNase I protection (positions −5 to −13; Fig. 5 and 6) and enhanced cleavage (position −14; Fig. 5 and 6) are not seen upstream of the tRNA^{Ser} gene, but nonetheless appear to be related to tRNA gene transcription, since they are dependent on TFIIIC binding. The existence of additional factors that interact with flanking regions has been suggested previously by in vitro transcription experiments. Both upstream and downstream sequences can influence tRNA gene transcription (12, 14, 40, 47) and have been shown in some cases to compete for transcription components in crude extracts (40, 47). The absence of cleavage alterations upstream of the tRNA^{Ser} gene could be accounted for by such components being either gene specific or displaying differential affinity for the flanking regions of different genes. Alternatively, the alterations could be due to RNA polymerase III interactions and be characteristic of initiation or preinitiation complexes of different stabilities. To distinguish between these possibilities it will be necessary to establish the full extent of the flanking region interactions and reproduce these phenomena in vitro.

A major concern in the nuclear footprinting studies is that cell lysis might disrupt the system to the extent that the observed DNase I cleavage patterns do not accurately reflect the in vivo state of the chromosomal genes. We cannot, for example, rule out the possibility that bound proteins are released from complexes with the gene during cell lysis or that factors are rebinding to the gene sequences, both of which would give artifactual representations of promoter occupation. To address this problem we are currently attempting to probe the chromatin structure with reagents capable of rapidly introducing base cleavages or modifications into the DNA of intact cells (4, 19). Although these techniques are also subject to limitations in terms of describing only static interactions and having potentially disruptive

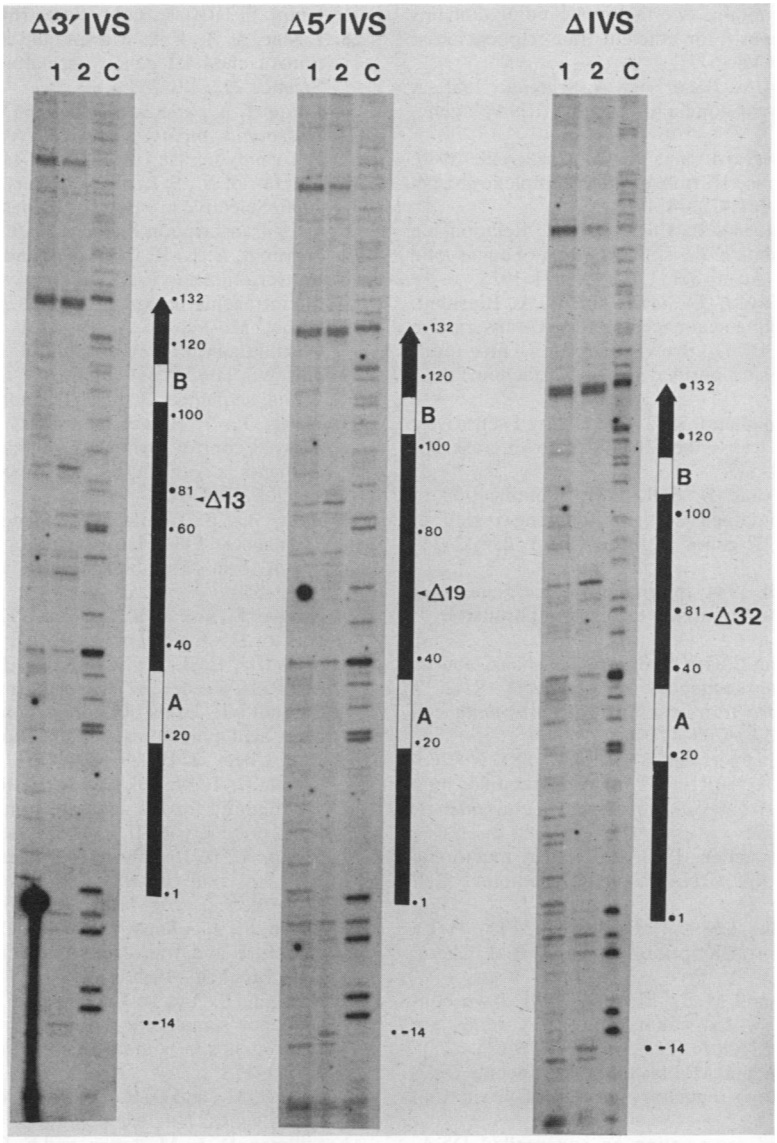

FIG. 6. DNase I footprinting of the chromosomal $tRNA^{Leu}_{SUP53}$ deletion variants. Cell lysates of the Δ3′IVS, Δ5′IVS, and ΔIVS DC5 transformants were prepared and treated as described in the legend to Fig. 5. Genomic DNA was probed with the oligonucleotide that hybridizes upstream of the $tRNA^{Leu}_{SUP53}$ gene. Lanes: 1 and 2, DNase I digestions of deproteinized and nuclear DNA, respectively; C, dideoxy C sequencing reactions with undigested genomic DNA from the respective transformant. The region encoding the primary transcript, the positions of the deletions and number of base pairs deleted are indicated to the right of each panel. Nucleotide positions are indicated at 20-base-pair intervals relative to the transcription initiation site. A band compression between positions 30 and 40 appears in this experiment, but is not reproducibly seen and does not affect the interpretation of the results.

effects, their use would provide an independent verification of the nuclear footprinting results.

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