MOLECULAR AND CELLULAR BIOLOGY, Oct. 1989, p. 4220–4228
0270-7306/89/104220-09$02.00/0

Vol. 9, No. 10

Mutations in the Anticodon Stem Affect Removal of Introns from Pre-tRNA in *Saccharomyces cerevisiae*†

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Received 2 March 1989/Accepted 10 July 1989

To evaluate the role of exon domains in tRNA splicing, the anti-codon stem of proline pre-tRNA$_{UGG}$ from *Saccharomyces cerevisiae* was altered by site-directed mutagenesis of the *suf8* gene. Sixteen alleles were constructed that encode mutant pre-tRNAs containing all possible base combinations in the last base pair of the anticodon stem adjacent to the anticodon loop (positions 31 and 39). The altered pre-tRNAs were screened by using an in vitro endonucleolytic cleavage assay to determine whether perturbations in secondary structure affect the intron excision reaction. The pre-tRNAs were cleaved efficiently whenever secondary structure in the anticodon stem was maintained through standard base pairing or G · U interactions. However, most of the pre-tRNAs with disrupted secondary structure were poor substrates for intron excision. We also determined the extent to which the *suf8* alleles produce functional products in vivo. Each allele was integrated in one to three copies into a yeast chromosome or introduced on a high-copy-number plasmid by transformation. The formation of a functional product was assayed by the ability of each allele to suppress the +1 frameshift mutation *his4-713* through four-base codon reading, as shown previously for the *SUF8-1* suppressor allele. We found that alleles containing any standard base pair or G · U pair at position 31/39 in the anticodon stem failed to suppress *his4-713*. We could not assess in vivo splicing with these alleles because the tRNA products, even if they are made, would be expected to read a normal triplet rather than a quadruplet codon. However, all of the alleles that contained a disrupted base pair at position 31/ 39 in the anticodon stem altered the structure of the tRNA in a manner that caused frameshift suppression. Suppression indicated that splicing must have occurred to some extent in vivo even though most of the suppressor alleles produced pre-tRNAs that were cleaved with low efficiency or not at all in vitro. These results have important implications for the interpretation of in vitro cleavage assays in general and for the potential use of suppressors to select mutations that affect tRNA splicing.

Splicing of transfer RNA in *Saccharomyces cerevisiae* requires three separate enzymes: a detergent-extractable endonuclease, a soluble ligase, and a 2′-phosphatase (26). The endonuclease cleaves the pre-tRNA, releasing the intron as a linear molecule (25). The ligase joins the half molecules through an ATP-dependent, three-step mechanism involving cyclic phosphodiesterase, polynucleotide kinase, and RNA ligase activities (12, 28). Finally, the residual 2′-phosphate at the splice site is removed to produce a mature, primary tRNA sequence.

The splicing enzymes of *S. cerevisiae* process all intron-containing intermediates in the synthesis of 10 of the 46 cytoplasmic tRNA isoacceptors (14, 23). The enzymes form an in vitro complex composed minimally of the endonuclease and the ligase (11). The ligase, and by extension probably the complex as well, is localized in vivo in association with the nuclear periphery or envelope (2). Since splicing is the last biochemical step in tRNA maturation before nuclear transport (22), localization of the complex in or near the nuclear envelope suggests the possibility of a direct relationship between splicing and export to the cytoplasm.

The endonuclease is one of the least-characterized components of the splicing complex. Endonuclease activity is required for the efficient removal of introns from pre-tRNA both in vitro (25) and in vivo (40). However, questions regarding its role in cleavage have recently been raised by the finding that autocatalytic cleavage at the 3′ intron-exon junction can occur under appropriate in vitro conditions in the absence of endonuclease or other proteins (38). To resolve the role of the endonuclease in the cleavage reaction, genetic studies have been initiated in which mutations affecting enzyme activity have been identified (40).

In addition to a genetic characterization of the endonuclease, we are also interested in defining the structural features of pre-tRNAs that promote accurate and efficient cleavage. The fidelity and efficiency of pre-tRNA cleavage is not dependent to any major extent on specific primary sequences surrounding the cleavage sites (13, 29, 39, 41). Instead, the three-dimensional conformation of the pre-tRNA appears to be much more important. All precleavage intermediates in *S. cerevisiae* fold into a consensus secondary structure that has the following unique features (17, 36): (i) the introns always interrupt the anticodon at the same position; (ii) the 5′ and 3′ cleavage sites reside in single-stranded regions of the precursor; (iii) introns participate in pre-tRNA secondary structure, the most common feature being the anticodon helix formed by base pairing between the intron and the 5′ exon; and (iv) the exon domains of

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pre-tRNA fold into a conformation resembling mature tRNA.

To examine how pre-tRNA conformation affects the cleavage reaction, we have investigated the extent to which intron excision is influenced by the exon domains that base pair to form the anticodon stem. We systematically altered the primary sequence of the anticodon stem by in vitro mutagenesis of the *SUF8* gene, one of five intron-containing genes that encode proline $tRNA_{UGG}$ (4). *SUF8* pre-tRNA was chosen for this analysis in part because its secondary structure has been described and conforms to the structural consensus typical of other *S. cerevisiae* pre-tRNAs (17). In addition, a previous study showed that the mutation *SUF8-1* ($G_{39} \rightarrow U_{39}$) reduces the efficiency of in vitro cleavage by disrupting the secondary structure of the anticodon stem (43).

In this study, 16 alleles of the *SUF8* gene were constructed that consist of single and double mutations at sites in the anticodon stem corresponding to position 31, position 39, or both. In the wild-type tRNA, these positions are normally occupied by nucleotides that form the last base pair of the anticodon stem adjacent to the anticodon loop. The mutant pre-tRNAs encoded by each allele were screened by using an in vitro cleavage assay to determine what effects disruption of secondary structure in the anticodon stem would have on endonucleolytic cleavage.

In a parallel set of experiments, each allele was assayed for the ability to confer frameshift suppression in vivo. Since suppression signals the formation of a functional product, it was possible in the case of those alleles that confer suppression to assess in vivo splicing in relation to the cleavage defects found in vitro.

MATERIALS AND METHODS

Genetic techniques. Genetic methods, nomenclature, and media for *Escherichia coli* and *Saccharomyces cerevisiae* have been described by Miller (20) and Gaber and Culbertson (10), respectively. *E. coli* 6507 (*pyr23*::Tn*5* *recA* Kan^r r_K^- m_K^- *leu pro*) and JM105 [Δ(*lac-pro*) *thi strA supE endA sbeB hsdR* F′ *traD36 proAB* $lacI^q$ *lac*Δ M15] were used for plasmid constructions and the isolation and analysis of in vitro-generated mutations. The methods of Petes et al. (27) and Ito et al. (15) were used to transform *E. coli* and yeast cells, respectively. Transfection with the replicative form of bacteriophage M13 was performed by the method of Messing (19).

DNA techniques. Recombinant DNA techniques, plasmid DNA purification, and the preparation of radioactive DNA probes by the method of nick translation are described by Maniatis et al. (18). Methods for preparation of M13 phage lysates and single- and double-stranded phage DNA are described by Messing (19). Yeast DNA used for hybridization by the method of Southern (34) was prepared as described by Craig and Jacobson (3).

***SUF8* cloning procedure.** *SUF8* alleles that confer frameshift suppression were cloned by a variation of the integration-excision procedure (30). The $suf8^+$ plasmid pCC14 (5) was linearized by *Sac*I digestion and integrated at the *SUF8* locus by transformation of recipient strains carrying the frameshift suppressor mutations *SUF8-2* through *SUF8-7*. Chromosomal DNA from a transformant of each recipient was digested with *Sal*I or *Bam*HI, ligated, and used to transform *E. coli* to ampicillin resistance. The resulting plasmids (pSUF8-2 through pSUF8-7) all contained a 455-base-pair *Xho*I fragment that carried the mutant *SUF8* alleles. The *Xho*I fragments were subcloned in M13mp18 for DNA sequence analysis by the method of Sanger et al. (32).

Oligonucleotide-directed mutagenesis. Two previously identified alleles of the *suf8* gene, $suf8^+$ and *SUF8-1* (4), were used as the initial substrates for gapped-duplex mutagenesis (9). They encode pre-tRNAs containing nucleotides C_{31}-G_{39} and C_{31}-U_{39}, respectively, according to the standard numbering system for tRNA as described by Schimmel et al. (33). In the wild-type tRNA, the nucleotides at positions 31 and 39 form the last base pair of the anticodon stem adjacent to the anticodon loop (Fig. 1). The mutagenesis protocol was designed to create all possible nucleotide combinations at positions 31 and 39.

The $suf8^+$ and *SUF8-1* genes were subcloned into the *Sal*I site of phage M13mp18 on a 455-base-pair *Xho*I fragment. The phages were then mutagenized as described below with deoxyoligonucleotides synthesized by the University of Wisconsin Biotechnology Center. The oligomer 5′-CAAAG CAT(**C/A**)CGAGAGGC-3′ was used to change U39 in *SUF8-1* to C and A (unique or mixed nucleotides at the site of mutation in the oligomer are indicated in boldface). To create nucleotide changes at position 31, phages encoding each of the four possible position 39 bases (C_{31}-U_{39} [*SUF8-1*], C_{31}-G_{39} [$suf8^+$], C_{31}-C_{39}, and C_{31}-A_{39}) were mutagenized with the oligomers 5′-GATTCTCG(**G/T/A**)TTTGGGCG-3′ and 5′-GATTCTCGTTTTGGGCG-3′. These procedures yielded 14 of the 16 possible mutants.

Two alleles expected from these procedures, G_{31}-A_{39} and G_{31}-U_{39}, were not recovered. The G_{31}-A_{39} allele was constructed by mutagenizing a phage encoding G_{31}-G_{39} with the first oligomer listed above to change position 39 from G to A. The G_{31}-U_{39} allele was constructed by mutagenizing a phage encoding C_{31}-U_{39} (*SUF8-1*) with the oligomer 5′-GATTC-TCGGTTTGGGCG-3′. This resulted in a change at position 31 from C to G.

Phages containing the desired mutations were identified by the plaque-screening assay of Zoller and Smith (44). The oligomers used for mutagenesis also served as probes in the screening procedure. DNA sequence analysis (32) was used to identify mutations of interest and to confirm that no other mutations were present in the genes after mutagenesis.

RNA techniques. End-matured pre-tRNAs encoded by each of the *SUF8* alleles were synthesized in vitro by using a yeast RNA polymerase III transcription system (8). Transcripts were labeled with [α-^{32}P]UTP (400 Ci/mmol; Amersham Corp.) in a reaction mixture containing nonradioactive ATP, CTP, and GTP. The pre-tRNAs were fractionated on 10% (wt/vol) polyacrylamide gels, eluted, and precipitated as described by Knapp et al. (16).

tRNA-splicing endonuclease was purified through fraction IV as described by Peebles et al. (25). Cleavage assays were performed as described by Winey et al. (43), using 0.1 U of enzyme per ml and approximately 1.5×10^{-8} g of pre-tRNA (30 nM). The pre-tRNAs remained intact, and no cleavage products were observed in reaction mixtures containing 1% Triton X-100 but lacking the fraction IV extract. Portions of 10 μl were removed from the reactions at various time points, and the reactions were terminated by the addition of 4 μl of a stop/loading buffer [10% [wt/vol] sodium dodecyl sulfate, 0.2 mM EDTA, 5 mg of proteinase K per ml, 0.05% [wt/vol] bromophenol blue and xylene cyanol FF, 10% [wt/vol] sucrose, 3.5 M urea). The reaction mixtures were then incubated at 50°C for 15 to 30 min and loaded directly onto 4 M urea–10% (wt/vol) polyacrylamide gels. Reaction products were visualized on the gels by autoradiography. Product yields were determined by Cerenkov counting of gel

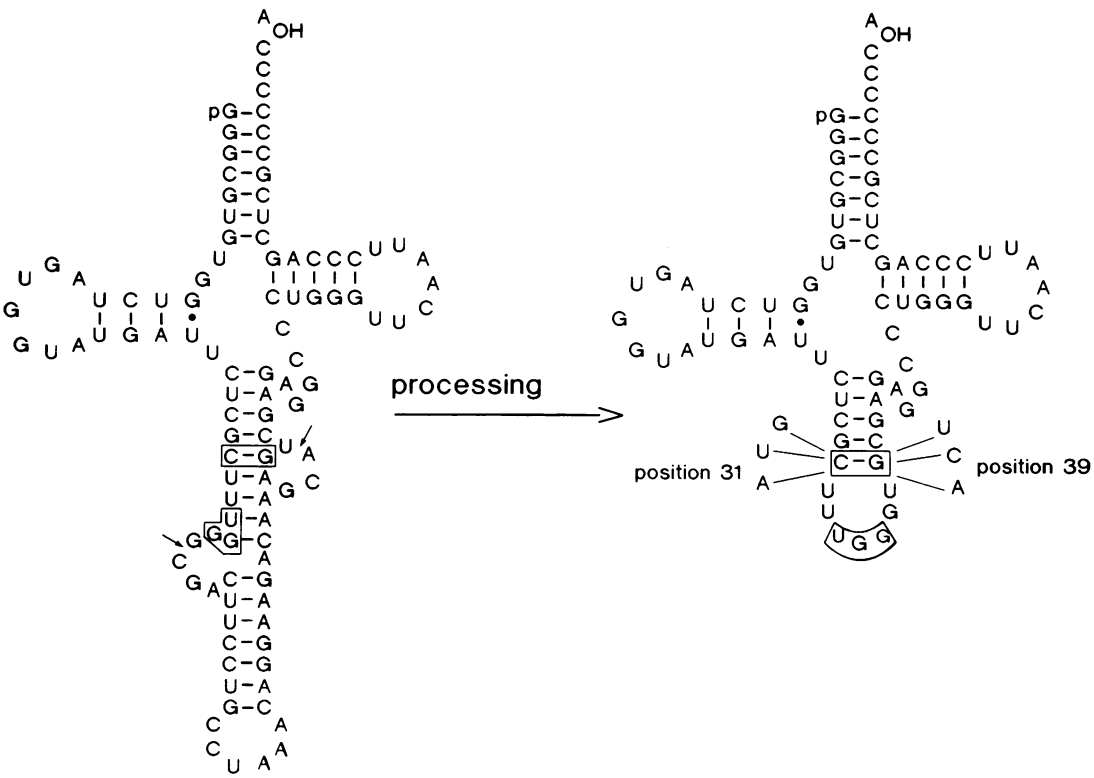

FIG. 1. Secondary structures for proline pre-tRNA$_{UGG}$ and mature proline tRNA$_{UGG}$. The secondary structure of the precursor was verified experimentally by Lee and Knapp (17). The mature tRNA is encoded by five genes, including *suf8* (42). The nucleotide sequences of the *suf8*$^+$ (C_{31}-G_{39}) and *SUF8-1* (C_{31}-U_{39}) alleles were determined by Cummins et al. (4). The RNA sequences, including modified nucleotides (not shown), were determined by Winey et al. (43). The mature tRNA is produced by enzymatic cleavage of a 31-base intron from the precursor at the sites indicated by arrows. The anticodon nucleotides of the mature tRNA and the corresponding nucleotides in the precursor are enclosed in boxes, as are nucleotides C_{31} and G_{39} in the anticodon stem of the mature tRNA and the corresponding nucleotides in the precursor. Positions 31 and 39 were changed to all possible alternative nucleotides by site-directed mutagenesis (see Materials and Methods).

slices in which the amount of radioactivity was normalized to the expected number of U residues in each RNA fragment.

To generate end-labeled pre-tRNAs, transcripts were prepared in vitro as described above except that nonradioactive UTP was substituted for [α-^{32}P]UTP. After fractionation, elution, and precipitation, the pre-tRNAs were 3′ end labeled with [α-^{32}P]ATP (3,000 Ci/mmol; Amersham), using yeast nucleotidyltransferase (17). The pre-tRNAs were used as substrates in the same cleavage reactions as described above except that the substrate concentrations were estimated to be in excess of 30 nM.

Partially purified nucleotidyltransferase was prepared as a by-product of tRNA-splicing endonuclease purification. Soluble proteins, including tRNA-splicing ligase and nucleotidyltransferase, were separated from yeast membranes by centrifugation. The supernatant, designated fraction II by Peebles et al. (25), was dialyzed against 30 mM potassium phosphate (pH 7.3)–1 mM EDTA–1 mM dithiothreitol (35). The tRNAs were removed from the dialysate by batch elution from DEAE-cellulose (Whatman DE-52) equilibrated in the dialysis buffer. The DEAE-cellulose was removed by filtration. The filtrate containing nucleotidyltransferase activity was judged to be free of endogenous RNA, since no labeled RNAs could be detected after a control reaction in which an exogenous RNA substrate was omitted.

Plasmid and strain construction. Frameshift suppression was examined in yeast cells after transformation with plasmids carrying mutations in the *suf8* gene. Integrative plasmids carrying the mutations were constructed by subcloning a 485-base-pair *Bam*HI-*Hin*dIII DNA fragment from phage M13mp18 into YIp5 (1). For some alleles, the same fragment was also subcloned into the multicopy, episomal 2 μm vector EC402 (a gift from E. Craig). EC402 is a derivative of YEp24 (1) in which the orientation of the 2 μm DNA was reversed.

YIp5 hybrid plasmids containing the *SUF8* alleles were integrated at the *ura3* locus on chromosome 5 by the method of Orr-Weaver et al. (24). The plasmids were linearized by digestion with *Eco*RV, which cleaves the plasmid DNA once at a site within the *URA3* gene (31). The linear plasmids were used to transform strain W153-10b (**a** *his4-713 lys2-20 ura3-52*) to a Ura$^+$ phenotype. Integration at the *URA3* locus was confirmed by analysis of tetrads from crosses between the transformants and strain W153-7a (α *his4-713 lys2-20 ura3-52 can1*^{r}). In all of the crosses, the integrated *URA*$^+$ gene was linked to the *can1*^{r} locus (21). Hybridization of YIp5 DNA to genomic yeast DNA from the transformants (34) was used to confirm the site of integration and to determine the number of integrated plasmid copies (see Results). Derivatives of the episomal plasmid EC402 were also introduced into strain W153-10b by transformation to a Ura$^+$ phenotype.

Assay for frameshift suppressor activity. The *his4-713* mutation in strain W153-10b causes a shift in the *his4* message to the −1 reading frame as a result of a +1C nucleotide insertion in a CCU proline codon (7). The *his4-713* mutation confers a His$^-$ phenotype, but this is sup-

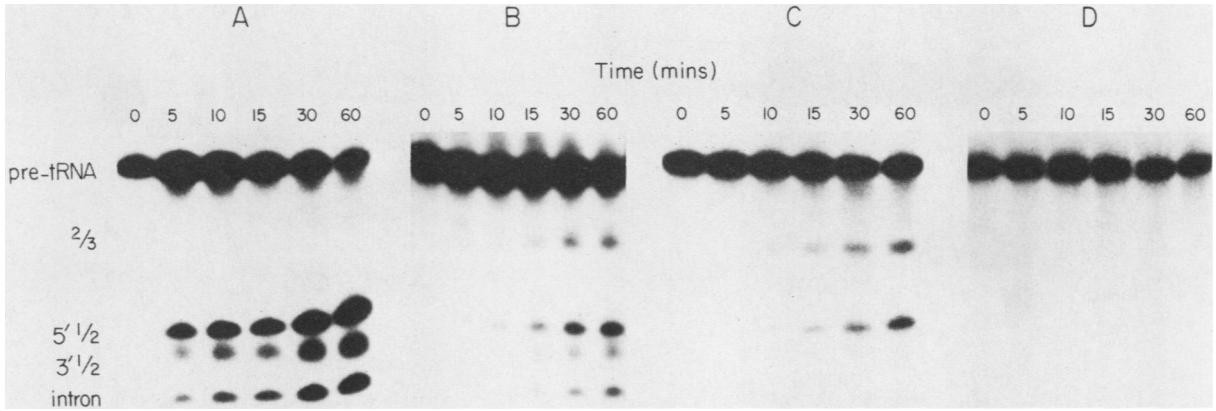

FIG. 2. In vitro cleavage of pre-tRNAs containing mutations in the anticodon stem. The figure shows the products resulting from cleavage over a 60-min time course at a substrate concentration of 30 nM, followed by fractionation on a 10% polyacrylamide gel and visualization by autoradiography. In some cases, different results were obtained by using higher substrate concentrations (see text). Band assignments in panel A were previously determined by Winey et al. (43). The assignments and amounts of products formed were also determined by 3' end labeling (Fig. 4) and by Cerenkov counting of gel slices in which the counts per minute was normalized to the number of radioactive U residues expected in each product (see Materials and Methods). Sixteen *SUF8* pre-tRNA substrates were assayed. They were divided into four groups on the basis of behavior in the cleavage reaction (see Table 1). Representative pre-tRNAs of each group are shown as follows: (A) C_{31}-G_{39} (*suf8*$^+$); (B) G_{31}-A_{39}; (C) C_{31}-U_{39} (*SUF8-1*); (D) C_{31}-C_{39}.

pressed to a His$^+$ phenotype in the presence of the *SUF8-1* frameshift suppressor (5). The *SUF8* alleles generated by site-directed mutagenesis in this study were assayed for in vivo function in the same manner, whereby suppression of *his4-713* was indicated by the growth of W152-10b transformants in the absence of exogenous histidine.

RESULTS

Site-directed mutagenesis of the *SUF8* gene. M13mp18 phages containing *SUF8* DNA were mutagenized with oligonucleotides (see Materials and Methods) to generate all possible base substitutions at positions 31 and 39 in the tRNA (Fig. 1). This resulted in a matrix of 16 position 31 and 39 base combinations. Of these, four had potential for base pairing, two had potential for G · U interactions, and the remainder lacked potential for standard interactions. This latter group was assumed to have a disruption in the secondary structure of the anticodon stem.

In vitro cleavage assays. Pre-tRNAs containing the mutations were assayed to determine their behavior in the intron excision reaction. The products resulted from two coincident reactions, 5' and 3' cleavage, yielding two half-tRNAs plus the intron (Fig. 2A). Intermediates (2/3 molecules) resulting from temporal decoupling of the cleavage reactions did not accumulate in appreciable yields with a wild-type pre-tRNA substrate (Fig. 2A). Thus, accumulation of 2/3 molecules in conjunction with disproportionate yields of the half-tRNAs and intron was considered primary evidence for noncoincident cleavages, indicating that the relative kinetics of 5' and 3' cleavage was altered by mutation of the substrate. By determining the relative amounts and types of reaction products, four groups of mutants (A through D) could be distinguished as described below. Representative results for each group are shown in Fig. 2A to D, respectively. The results for all of the substrates are summarized in Table 1.

Group A. Coincident 5' and 3' cleavage was observed for 8 of the 16 pre-tRNAs: C_{31}-G_{39} (wild type), G_{31}-C_{39}, A_{31}-U_{39}, U_{31}-A_{39}, G_{31}-U_{39}, U_{31}-G_{39}, A_{31}-C_{39}, and G_{31}-G_{39}). For each pre-tRNA, there were no appreciable yields of 2/3 molecules. Equimolar yields of the 5' half-tRNA, 3' half-tRNA, and intron were obtained. The identities of these products were determined previously (43). The reaction products reached near maximum levels of accumulation by 30 min, at which time approximately 20 to 30% of the precursor had been utilized.

Overall, the group A mutant substrates exhibited behavior comparable to that of the wild-type pre-tRNA in the in vitro cleavage reaction. Thus, the mutations of group A appeared to have negligible effects on intron removal in vitro.

Group B. Cleavage assays of four pre-tRNAs (G_{31}-A_{39}, A_{31}-G_{39}, C_{31}-A_{39}, and U_{31}-U_{39}) resulted in the accumulation of a 2/3 intermediate in addition to the 5' half-tRNA, 3' half-tRNA, and intron. Essentially the same results were obtained when substrate concentrations were increased from 30 to 100 nM. Analysis of the normalized amount of radioactivity in each band revealed a reduced molar yield of the 3' half-tRNA and intron relative to the 5' half-tRNA. This result suggested that the band migrating in the size range for 2/3 molecules is the uncleaved 3' half-tRNA plus intron. This conclusion was supported by additional criteria. First, the quantitative data suggested that the appearance of the 3' half-tRNA lagged somewhat behind that of the 5' half-tRNA, as would be expected in the case of reduced 3' cleavage efficiency. Second, the reduced molar yield at each time point for the 3' half-tRNA and intron could be fully accounted for by the appearance with time of an equivalent molar yield of the 2/3 molecule. In addition to these effects, the overall utilization of class B substrates was somewhat lower than that of class A substrates (10 to 15% compared with 20 to 30% in 30 min).

Group C. The pre-tRNA encoded by the original *SUF8-1* allele (C_{31}-U_{39}) was unique in its effects on the cleavage reaction. Virtually all of the radioactivity not found in the uncleaved precursor appeared in two bands previously identified as the 3' 2/3 molecule and the 5' half-tRNA (38). No appreciable yields of the 3' half-tRNA or intron were obtained. However, when the substrate concentration was increased from 30 to 100 nM, small but detectable amounts of the 3' half-tRNA and intron were observed (not shown in

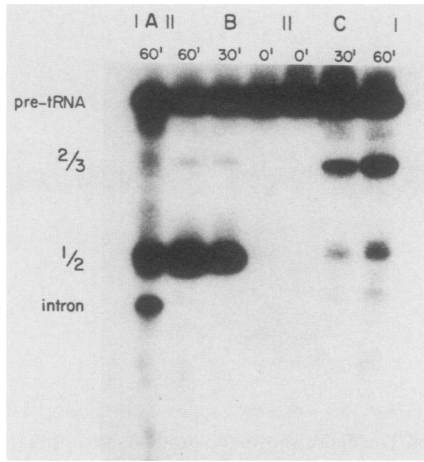

FIG. 3. Identification of cleavage products by 3′ end labeling. Pre-tRNA transcripts were 3′ end labeled with [α-^{32}P]ATP, using yeast nucleotidyltransferase (see Materials and Methods). The end-labeled pre-tRNAs were then used as substrates in the cleavage reaction. Since only the products containing the original 3′ end of the pre-tRNA are labeled, the procedure permits the assignment of half and 2/3 molecules. Panel A, Control showing the half- and 2/3-tRNA cleavage products obtained by using a wild-type pre-tRNA substrate uniformly labeled at all U residues (similar to that shown in Fig. 2). Panel B, A 60-min time course, using 3′-end-labeled *suf8*$^+$ pre-tRNA (C_{31}-G_{39}) as the substrate. The labeled bands correspond to the uncleaved pre-tRNA and the 3′ half-tRNA. Panel C, A 60-minute time course, using 3′-end-labeled *SUF8-1* pre-tRNA (C_{31}-U_{39}) as the substrate. The labeled bands correspond to the uncleaved pre-tRNA, the 3′ 2/3 molecule, and a relatively small amount of the 3′ half molecule that appears when the substrate concentration exceeds 30 nM. This result confirms that the 2/3 molecule accumulates as a result of inefficient cleavage at the 3′ intron-exon junction. Results similar to that shown in panel C were obtained with the group B pre-tRNA substrates G_{31}-A_{39}, C_{31}-A_{39}, and U_{31}-U_{39}.

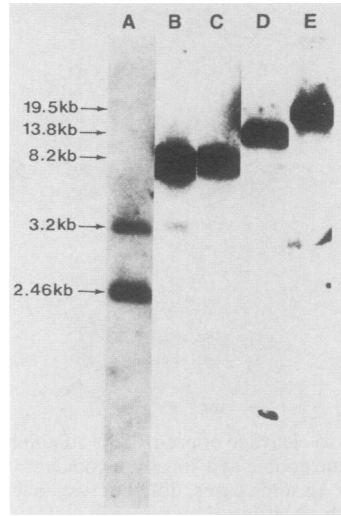

FIG. 4. Determination of gene copy number in yeast transformants. To assay for expression in vivo, *SUF8* alleles constructed in vitro were subcloned into YIp5 (1) and integrated at the *ura3* locus on chromosome 5 by yeast transformation (see Materials and Methods). In the representative experiments shown, radioactively labeled YIp5 plasmid DNA was used to probe genomic *Bgl*II-digested DNA from transformants of strain W153-10b (see text). Lane A, Control showing hybridization of YIp5, which contains the *URA3* gene, to DNA from the untransformed recipient. Hybridization to the 3.2-kb band is weak and variable because of the limited extent of homologous overlap between the probe and the 3.2-kb fragment. The 2.46-kb band contains an *Eco*RV site into which the plasmids were integrated. The number of integrated plasmid copies was determined by the extent to which the 2.46-kb band increased in size. Since each plasmid is 6.026 kb in length, the theoretical band sizes expected for one, two, and three plasmid copies are 8.486, 14.512, and 20.538 kb, respectively. The fragment sizes shown were determined empirically by comparison with the positions of migration of bacteriophage λ *Hin*dIII fragments of known size (not shown). The 8.2-kb bands in lanes B and C indicate integration of a single plasmid copy; the 13.8- and 19.5-kb bands in lane D and E indicate integration of two and three plasmid copies, respectively.

Fig. 2C). The precursor was utilized in the reaction to an extent resembling results for the class B substrates (10 to 15% in 30 min). Overall, the results indicate a pronounced reduction in 3′ cleavage efficiency and a much lesser effect on 5′ cleavage efficiency.

Group D. Three pre-tRNAs (U_{31}-C_{39}, C_{31}-C_{39}, and A_{31}-A_{39}) were poor substrates for endonuclease cleavage at both 5′ and 3′ intron-exon junctions. Essentially all of the radioactivity was found in the uncleaved precursor, and no detectable intermediates or products were formed. The same results were obtained when the substrate concentration in each reaction was increased from 30 to 100 nM. These pre-tRNAs are therefore defective for in vitro cleavage at both the 5′ and 3′ intron-exon junctions.

Cleavage of 3′-end-labeled pre-tRNA. Results of the experiments described above suggested that the 2/3 molecules of groups B and C represented uncleaved introns fused to 3′ half-tRNAs. Further support for this conclusion was obtained by determining which products accumulated in cleavage reactions containing pre-tRNAs labeled at the 3′ end (Materials and Methods; Fig. 3). Since this approach provides a way to assess the distribution of 3′ ends among the products, the identities of the 2/3 and half molecules could be unambiguously assigned.

The wild-type group A pre-tRNA (C_{31}-G_{39}) yielded only one prominent, labeled product that migrated in the region of the half-tRNAs (Fig. 3, panel B). By contrast, cleavage of the group C pre-tRNA (C_{31}-U_{39}) yielded two labeled products, one that migrated in the region of half-tRNAs and another that migrated in the region of the 2/3 molecules (Fig. 3C). By virtue of the position of the label at the 3′ end of the precursor, one product was the 3′ half-tRNA, which, as expected, was present in low yield, and the other was the 3′ 2/3 molecule (uncleaved intron plus 3′ half-tRNA) (Fig. 3C). Similar results (not shown) were obtained for three other group B pre-tRNAs (G_{31}-A_{39}, C_{31}-A_{39}, and U_{31}-U_{39}). One group B pre-tRNA (A_{31}-G_{39}) proved to be a poor substrate for nucleotidyltransferase and was not assayed by this method. These results provide an explanation for the observed temporal decoupling of the two cleavage reactions: the efficiency of cleavage at the 3′ intron-exon junction is preferentially reduced compared with the efficiency of 5′ cleavage.

In vivo expression of *SUF8* alleles. The ability of *SUF8* alleles to produce functional products in vivo was assayed by determining whether the genes conferred frameshift suppression after transformation into yeast cells (see Materials and Methods). To better evaluate the results of suppression tests, transformants of strain W153-10b were first analyzed by hybridization, using the method of Southern (34), to determine the number of chromosomally integrated plasmid copies (Fig. 4). The linear plasmids used for transformation

TABLE 1. Summary of in vitro cleavage and suppression assays

SUF8 allele[a]	In vitro cleavage[b]			Suppression[c]			
	Cleavage group	Cleavage site affected		With given no. of chromosomal gene copies			With episomal (2μm) multiple copies
		5′	3′	1	2	3	
C-G	A	No	No	−	−	−	−
G-C	A	No	No	−		−	
A-U	A	No	No	−	−		
U-A	A	No	No	−	−		
G U	A	No	No	−	−		
U G	A	No	No	−	−		
G G	A	No	No	−	−		+
A C	A	No	No	+		+	
G A	B	No	Yes	−	+		
A G	B	No	Yes[d]	−	−		+
C A	B	No	Yes	−			+
U U	B	No	Yes	−	+		
C U	C	No	Yes	++	++	++	+++
U C	D	Yes	Yes	+	+		
C C	D	Yes	Yes	−		+	
A A	D	Yes	Yes	−	+		

[a] Alleles are designated according to nucleotides occupying positions N_{31} and N_{39} in the anticodon stem (see Fig. 1). Nucleotide substitutions are shown in boldface.

[b] Groups A to D refer to the classification of alleles based on in vitro cleavage assays (Fig. 2).

[c] The copy number of genomic integrants in strain W153-10b was determined by hybridization, using the method of Southern (34). In addition, some alleles were subcloned into the multicopy 2μm plasmid EC402 and tested for suppression after transformation into strain W153-10b (see Materials and Methods). Suppression of the frameshift mutation *his4-713* (7) was determined by assaying growth on petri plates lacking histidine. +++, Visible growth in 1 to 2 days; ++, visible growth in 2 to 3 days; +, visible growth to 4 to 10 days; −, no growth. A blank space indicates that no test was performed.

[d] As judged only by relative product yields. We were unable to 3′ end label this substrate by using nucleotidyltransferase.

integrate within a 2.46-kilobase (kb) *Bgl*II fragment containing the *ura3-52* gene (31). Since the plasmids contain no *Bgl*II sites, hybridization of YIp5 DNA to *Bgl*II-digested yeast DNA from each transformant provides a diagnostic test for the number of integrated plasmid copies. Hybridization to the 8.2-, 13.8-, and 19.5-kb bands shown in Fig. 4 indicated single, double, and triple integration events. None of the transformants analyzed contained more than three integrated plasmid copies.

We assessed the structural integrity of multiple plasmid integrants by digesting DNA from each transformant with *Eco*RV and probing gel-separated fragments with radioactively labeled YIp5 DNA (34). In all cases, only one band was obtained that comigrated with the original *Eco*RV-digested plasmid used for transformation. This result shows that the integrated plasmid sequences are structurally intact in multiple integrants. It is therefore likely that the multiple copies of the *SUF8* gene in each transformant are all functional.

Suppression was monitored by determining whether transformants of strain W153-10b could grow in the absence of exogenous histidine (Table 1; see Materials and Methods). Only three alleles (A_{31}-C_{39}, C_{31}-U_{39}, and U_{31}-C_{39}) conferred a His^+ phenotype when present in a single chromosomal copy. Among these, the original *SUF8-1* mutation (C_{31}-U_{39}) was the most efficient suppressor. In strains carrying two chromosomal gene copies, three additional alleles (G_{31}-A_{39}, U_{31}-U_{39}, and A_{31}-A_{39}) conferred a His^+ phenotype. In the presence of three gene copies, one additional allele (C_{31}-C_{39}) conferred a His^+ phenotype. Finally, three *SUF8* alleles (G_{31}-G_{39}, A_{31}-G_{39}, and C_{31}-A_{39}) conferred a His^+ phenotype only when introduced into strain W153-10b on a multicopy 2-μm plasmid.

By contrast, a strain carrying the wild-type allele (C_{31}-G_{39}) was His^- even when present on a multicopy 2 μm vector. We also tested 50 to 100 integrative transformants for each of the first six alleles listed in Table 1. None of these grew after 10 days of incubation on medium lacking histidine. Since about 15% of all transformants analyzed by hybridization contained three gene copies, we conclude that none of these alleles are likely to confer suppression when present in three gene copies or less.

Since only three of the in vitro-generated *SUF8* alleles conferred suppression in one gene copy, an experiment was performed that enabled a comparison with *SUF8* frameshift suppressors identified by an in vivo screen. Six previously isolated *SUF8* suppressors of independent origin (*SUF8-2* through *SUF8-7*) (4) were cloned, and the DNA sequences were determined (see Materials and Methods). Of these, five were isolated as spontaneous revertants and one was isolated as a UV-induced revertant of the frameshift mutation *his4-713* (a +1C insertion in a CCU proline codon) (7). DNA sequence analysis revealed that all six mutations were identical to the original *SUF8-1* allele (C_{31}-U_{39}). Thus, the in vivo screen uncovered the most efficient suppressor, but other less efficient suppressors, including two alleles, A_{31}-C_{39} and U_{31}-C_{39}, that were shown to confer suppression when present in a single gene copy, were not represented.

In summary, our results show that 10 of the 16 in vitro-generated *SUF8* alleles confer frameshift suppression in vivo. By contrast, only 1 of the 16 alleles, which conferred the highest efficiency of suppression, was recovered in an in vivo screen for suppressors.

DISCUSSION

In vitro cleavage defects. Pre-tRNAs encoded by the *SUF8* alleles were divided into four groups on the basis of behavior in an in vitro cleavage assay. Eight pre-tRNAs in group A were cleaved in a manner similar to that of the wild type. Four pre-tRNAs in group B and one in group C were cleaved inefficiently at the 3′ cleavage site. Three pre-tRNAs in group D were cleaved inefficiently at both the 5′ and 3′ cleavage sites. One potential group was conspicuously absent in that none of the pre-tRNAs were specifically defective for 5′ cleavage.

In theory, the mutations could cause one of two types of defect: inefficient cleavage or cleavage at incorrect sites. Eight of the altered pre-tRNAs were cleaved less efficiently than was the wild type (Fig. 2 and 4). Among these, five were predominantly affected at the 3′ intron-exon junction. One allele has been analyzed in greater detail to determine the exact sites of endonuclease cleavage. It was shown by RNA sequence analysis that the mature, in vivo product of the *SUF8-1* gene (group C, C_{31}-U_{39}) is produced as the result of cleavages at the same 5′ and 3′ sites as in the wild-type tRNA (43). Furthermore, a comparison of *SUF8-1* alleles either containing or lacking an intron suggested that the tRNA product derived by splicing acts as the sole agent of suppression. No minor products resulting from ragged, incorrect cleavages were detected in vivo that might have conferred suppression, nor were any minor products detected in vitro. Therefore, incorrect cleavages do not occur in the case of the group C mutation, *SUF8-1*. Although the other alleles were not analyzed to the same extent, none of the pre-tRNAs yielded minor products due to ragged, incor-

rect cleavages. There is no indication from the data obtained that these pre-tRNAs result in fidelity errors.

Cleavage is influenced by secondary structure in the anticodon stem. There is a pronounced preference in the cleavage reaction for nucleotides at positions 31 and 39 that are capable of interacting through the formation of hydrogen bonds. In general, pre-tRNAs that maintain normal secondary structure in the anticodon stem show little or no perturbations in the cleavage assay. However, most of the pre-tRNAs that lack the potential for standard interactions between nucleotides at positions 31 and 39 have adverse affects on the cleavage reaction.

Changes in the secondary structure of the anticodon stem might affect cleavage in at least two ways. First, the helical configuration of the anticodon stem is reduced in length from five to four base pairs. Second, the length of the single-stranded loop that contains the 3′ cleavage site is increased by one nucleotide. Increasing the length of the cleavage loop may distort the substrate in the vicinity of the 3′ cleavage site and account for some cleavage defects. In other cases, general distortion of the helix in the anticodon stem may influence cleavage at the 3′ site, the more distal 5′ site, or both.

Two exceptional substrates, G_{31}-G_{39} and A_{31}-C_{39}, exhibited behavior inconsistent with this interpretation. These substrates were cleaved in vitro in a manner resembling that found for the wild type (C_{31}-G_{39}) but contained nucleotides at position 31 and 39 that lacked potential for standard interactions. As discussed at greater length below, both of these pre-tRNAs give rise to functional frameshift suppressor tRNAs in vivo. Since suppression and altered cleavage are both consequences of disrupted base pairing between nucleotides 31 and 39, the mutations in these substrates should not in theory confer suppression without affecting the cleavage reaction. Thus, these nucleotide combinations cause structural perturbations that are unique and at present inexplicable. We view them, however, as exceptions to an otherwise valid rule of preference that explains the vast majority of the data.

We also searched for additional nucleotide preference rules by grouping the substrates according to several different criteria and then comparing the behaviors of substrates within the groups. We examined four groups: (i) reciprocal pairs (e.g., X_{31}-Y_{39} versus Y_{31}-X_{39}); (ii) single-site mutations at position 31 or 39; (iii) purine-purine, pyrimidine-pyrimidine, and purine-pyrimidine nucleotide combinations at positions 31 and 39; and (iv) substrates grouped according to the proximity of mutations in the linear RNA sequence to one cleavage site or the other.

No preference rules were revealed in this analysis that compared in importance to the requirement for base pairing in the anticodon stem. However, some less pronounced effects were uncovered. For example, among six reciprocal pairs, two contain reciprocal members that have dissimilar effects on cleavage (C_{31}-U_{39}–U_{31}-C_{39} and A_{31}-C_{39}–C_{31}-A_{39}). Even though the same nucleotides are present in each of these reciprocal pairs, pre-tRNA structure might be affected differently due to their opposite orientations. For example, they might have different effects on stacking interactions with neighboring nucleotides.

In addition, there were relatively fewer or less severe cleavage defects among alleles containing G_{31} than among alleles containing other position 31 nucleotides, including those that contain the C_{31} nucleotide found in the wild type. This preference holds true even where base pairing is not possible, as in the case of G_{31}-G_{39}, which is cleaved normally.

In vivo expression of *SUF8* alleles. Frameshift suppression was used to assess the expression of the *SUF8* alleles in vivo. Three criteria must be satisfied in order for frameshift suppression to occur. First, the intron must be removed and a mature tRNA produced at a sufficient but not necessarily optimal rate through the cleavage and ligation reactions. Second, the *SUF8* mutations cannot impose a complete block at any other step in tRNA biosynthesis, transport, or function. Finally, the product must satisfy the tRNA structural requirements that promote suppression. Since multiple criteria must be satisfied in order for suppression to occur, insight regarding tRNA splicing and frameshift suppression is most evident in cases where suppression is observed. In other cases, negative results are less easily interpreted without further analysis. Overall, the results of the suppression tests are instructive from several points of view, as discussed below.

There is a direct relationship between the types of structural perturbations in the anticodon stem and the ability of the gene products to suppress the *his4-713* frameshift mutation. Suppression occurs only when the potential for hydrogene bonding between nucleotides at positions 31 and 39 is lost as the result of base substitution. In the six cases where hydrogen bonding is possible (Table 1), no suppression was observed. Since these pre-tRNAs are cleaved normally in vitro, they may produce functional products that read normal triplet rather than quadruplet codons. In the remaining 10 pre-tRNAs, in which hydrogen bonding is disrupted, suppression results from an expansion of the anticodon loop from seven to nine nucleotides. There are two possible ways that the tRNAs might read a four-base codon and result in suppression. The tRNAs may read a four-base codon 5′-CCAA-3′ within the *his4-713* frameshift window by utilizing a four-base anticodon, 3′-GGUU-5′, as proposed by Curran and Yarus (6). Alternatively, the tRNAs may read a 5′-CCCU-3′ codon at the site of the frameshift in *his4-713* by utilizing the four-base anticodon sequence 3′-GGGU-5′ within the anticodon loop, as proposed by Winey et al. (43) (Fig. 1).

In those cases in which the *SUF8* alleles confer suppression, the corresponding pre-tRNAs must have been spliced in vivo in order to produce a functional product. In accordance with this reasoning, we compared the in vitro cleavage results with the ability to produce functional suppressors in vivo. The analysis is not, however, without complications, because the same perturbations in anticodon stem structure that promote frameshift suppression also appear to result in endonuclease cleavage defects that tend to limit suppression. The efficiencies of suppression therefore represent a trade-off between the amount of product produced by splicing and the structural features of the product that allow it to act as a frameshift suppressor. The suppression assays are clearly not as sensitive as a direct in vivo splicing assay, which has yet to be developed, but they do provide a first glance at the in vivo behavior of pre-tRNAs that show cleavage defects in vitro.

The extent to which each allele conferred suppression varied over a wide range, as indicated by a dependence on gene dosage. Three alleles conferred detectable suppression when present in a single dose, whereas at the other extreme, three different alleles conferred suppression only when present on a multicopy plasmid. An in vivo screen for *SUF8* suppressor mutations yielded similar results in that only

SUF8-1 (C_{31}-U_{39}), the most efficient of all the suppressors, was recovered.

There is no simple correlation between the extent of cleavage defects in vitro and the efficiencies of suppression in vivo (Table 1). Most noteworthy are alleles such as G_{31}-G_{39} (group A) or A_{31}-G_{39} (group B), which either are cleaved normally in vitro or suffer minimal cleavage perturbation but which rank among the least efficient suppressors. In these cases, inefficient suppression may result from negative effects of the mutations on aspects of tRNA biosynthesis, transport, or function other than intron cleavage. For example, it has been shown that the nucleotide at position 39 influences the rate of tRNA transport from the nucleus to the cytoplasm in *Xenopus* oocytes (37). Thus, transport might be adversely affected in some of the mutants.

Comparison of the cleavage and suppression data also reveals that some pre-tRNAs that have severe cleavage defects in vitro produce efficient suppressors in vivo. For example, in group C the C_{31}-U_{39} substrate yields no detectable 3′ half-tRNA in vitro, yet it is the most efficient of all the suppressors. Also, in group D the U_{31}-C_{39} substrate is not cleaved at all in vitro despite the fact that it is among the three most efficient suppressors. These discrepancies may indicate that the conditions for in vitro cleavage differ from the in vivo reaction conditions. For example, a 30 nM concentration of the *SUF8-1* substrate (C_{31}-U_{39}) in the in vitro cleavage reaction yields no 3′ half-tRNA (Fig. 2), whereas some 3′ half-tRNA is formed in vitro at a higher substrate concentration. This result indicates that if the local pre-tRNA concentration is high enough in vivo, both half-tRNAs might be produced in sufficient amounts to generate a mature suppressor tRNA. Furthermore, we showed previously that the *SUF8-1* (C_{31}-U_{39}) pre-tRNA yields some mature tRNA in a coupled endonuclease-plus-ligase assay even under conditions in which no 3′ half-tRNA is formed in the cleavage assay alone (43). One possibility is that the endonuclease functions at optimal efficiency as part of an active splicing complex (11) and that the in vitro cleavage assay used in these studies measures activity in extracts containing a partially active complex.

The comparison of in vitro cleavage and in vivo suppression data has important implications for biochemical and genetic analyses of tRNA splicing. The results indicate that mutations can affect splicing differently under standard in vitro cleavage conditions than actually occurs in vivo. This conclusion would have been more difficult to argue for any one mutation, but it becomes increasingly persuasive upon analysis of a larger number of related mutations such as those described in this study. The results suggest caution in the interpretation of any single mutation that affects the in vitro processing of tRNA introns. While valid general conclusions can be gleaned from existing approaches, our studies highlight a need for future improvements in the in vitro system and the development of in vivo cleavage and ligation assays.

In addition, the results make an important point regarding the use suppressors to screen for mutations affecting tRNA processing. In general, mutations that reduce the efficiency of intron removal in vitro might be expected to limit the production of suppressor tRNA in vivo. In theory, this provides a basis for selection of mutations that restore normal suppression through correction of the splicing defect. However, this rationale assumes a reasonable correlation between the rate of in vitro splicing, the formation of product in vivo, and the efficiency of suppression in vivo. Our data indicate that the necessary correlations may not exist and may explain the general lack of success in using suppressors to identify *trans*-acting factors required for tRNA splicing.

Role of the anticodon stem in the removal of introns. Several other studies support the view that the anticodon stem plays a pivotal role in the removal of introns. Most noteworthy among these are two reports showing that increasing the length of the anticodon stem alters the fidelity of the cleavage reaction (13, 29). For example, a double mutant of pre-tRNA^{Phe} containing a base-pair insertion in the anticodon stem, resulting in six rather than five base pairs, affects fidelity at both cleavage sites (29). The intron excised from this precursor in vitro was found to be one nucleotide longer at each end, suggesting that the stem provides a metric in cleavage site selection.

The mutant pre-tRNAs described in this study that contain base-pair disruptions suffer a decrease in the length of the anticodon stem from five to four base pairs. One mutation of this type, *SUF8-1* (C_{31}-U_{39}), has been shown definitively not to affect cleavage fidelity. This mutation, however, differs from the insertion mutations in that the length of the anticodon loop is increased and may compensate for a shortened stem. Overall, the results suggest that the metric for cleavage site selection includes not only the anticodon stem but also at least some portion of nucleotides in the anticodon loop.

The general picture emerging from these studies is that the anticodon stem serves the function of a structural linker that spans between two functionally distinct regions of the substrate. These regions probably interact with different parts of the endonuclease. A simple model consistent with all of the data is that the enzyme binds to the exon domains of the substrate in a manner that aligns one or more active sites in the enzyme with the cleavage sites in the substrate. Nucleotides in the anticodon stem-and-loop region provide a yardstick to locate the cleavage sites. Thus, mutations that fail to preserve length alter cleavage fidelity. Our studies indicate that in addition to depending on length, efficient cleavage also depends on the precise conformation and orientation of the anticodon stem and loop relative to other parts of the substrate.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, by U.S. Public Health Service grant GM40310 from the National Institutes of Health (M.R.C.), by National Science Foundation grant DCB8068054 (M.R.C.), and by U.S. Public Health Service training grant in molecular and cellular biology GM07215 from the National Institutes of Health (M.W.). Oligonucleotide synthesis performed by the University of Wisconsin Biotechnology Center was supported by Public Health Service grants SEG-S10-RR01684 and NCI-CA-07175 from the National Institutes of Health.

We thank David Engelke for assistance in the preparation of POLIII transcription extracts and Mark Mandel for technical assistance.

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