Purines Are Required at the 5' Ends of Newly Initiated RNAs for Optimal RNA Polymerase III Gene Expression

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We have made specific alterations in the CAACAA element at the transcription start site of a *Saccharomyces cerevisiae* suppressor tRNA gene. The mutant genes were tested for their ability to suppress the ochre nonsense alleles *ade2-1*, *lys4-1*, and *met4-1*. Many of the mutants showed either no phenotypic change or a weak loss of suppression relative to that of *SUP4-o*. A 2-bp change, CTCCAA, which alters bases encoding the +1 and +2 nucleotides of pre-tRNA^{Tyr}, had a strong deleterious effect in vivo, as did the more extensive change CTCCTC. In contrast, mutant genes bearing each of the possible single changes at nucleotide +1 retained normal suppression levels. The transcription start point could be shifted in a limited fashion in response to the specific sequences encountered by RNA polymerase III at the start site. ATP was preferentially utilized as the 5' nucleotide in the growing RNA chain, while with start site sequences that precluded utilization of a purine, CTP was greatly preferred to UTP as the +1 nucleotide. Short oligopyrimidine RNAs formed on the CTCCTC allele could be repositioned in the active center of the newly formed ternary complex. Early postinitiation complexes containing short nascent RNAs formed on the CTCCTC mutant were more sensitive to the effects of heparin and produced more abortive transcripts than similar complexes formed on *SUP4-o*. Our results suggest that the purine-rich sequences at the 5' ends of the nascent transcripts of many genes act to stabilize the early ternary complex.

The faithful and efficient transcription of DNA into RNA is a primary regulatory point in gene expression. The relative rates at which different genes are transcribed by the same RNA polymerase are strongly influenced by sequences surrounding the transcription start site. Previous studies of these contextual effects have emphasized sequence differences in the formation of the initiation complex and in the selection of the initiation site. RNA polymerases, both bacterial and eukaryotic, preferentially initiate transcription with a purine residue (4, 5, 8, 14, 18, 29, 30). In contrast, pyrimidine-specific transcription start sites are far less frequent as judged by 5'-end determination of RNAs synthesized both in vivo and in vitro (5, 18); only 7% of 88 *Escherichia coli* promoters surveyed by Hawley and McClure (14) directed transcription start sites with either UTP or CTP, with pyrimidine-specific initiation occurring only in the absence of purines within a defined window (36). The specific binding of purines at the *i* site of *E. coli* RNA polymerase, both in the presence and in the absence of template DNA, suggests that the interaction of purine ribonucleotides with RNA polymerase is important for productive transcription initiation (1, 47–49). However, the precise role in this process of nucleic acid sequences at and immediately following position +1 remains unclear.

As a system for studying small or large quantitative effects upon the level of transcription of a gene, RNA polymerase III transcription of the *SUP4* gene in the yeast *Saccharomyces cerevisiae* is highly advantageous. With different UAA alleles of yeast structural genes as reporters, mutations that change the in vivo level of mature tRNA^{Tyr} are detectable over a broad range. These same mutant *SUP4* genes can then be used as transcription templates in a yeast in vitro polymerase III extract to identify the one particular step in transcription that is

defective. Any phenotypic effects of *SUP4* mutations located 5' to the tRNA coding region may be ascribed to changes in transcription rather than altered RNA processing, as the first 12 nucleotides of the *SUP4* primary transcript are removed by an RNase P activity (9, 26) that does not depend on these sequences for its activity (6, 32, 46).

Consensus polymerase III initiation sequences have been determined from alignment of the 5'-flanking regions of yeast tRNA genes; within the conserved elements the sequence around the start site from –1 to +5 was CA₊₁ACAA in one study (39) and CA₊₁(A/T)AA in another (19). These conserved elements are similar to proposed RNA polymerase II initiation sites (13, 15, 33), which suggests functional similarities between the two systems. In particular, purine residues are greatly preferred at positions early in the transcript for both polymerases II and III. Gross terminal deletions have been used to functionally map the essential 5'-flanking sequences of eukaryotic class III genes (extensively reviewed in reference 43). These studies suffer from the general uncertainty arising from the creation of novel DNA junction sequences, and the traditional assay for lowered transcription efficiency provides no clue as to the functional lesions produced by these mutations. Recently, specific DNA sequence changes produced within the conserved sequence element at the transcription start site of the yeast *LEU3* gene have confirmed the importance of this element for optimal in vitro transcription (11).

In this report, we explore the role that sequences at and near the transcription start site play subsequent to transcript initiation. In particular, we look at the in vivo and in vitro effects of replacing purine bases with pyrimidines in the first two positions of the transcript. The results demonstrate the importance of purines at the initiation site for optimal gene expression both in vivo and in vitro. Newly formed transcription complexes that initiate transcription with pyrimidine residues are less stable than those starting with purines, suggesting a mech-

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anistic rationale for the preferential use of purines as the 5'-terminal nucleotide during transcription.

MATERIALS AND METHODS

Chemicals and enzymes. Reagents for buffer preparation were of molecular biology grade. Ultrapure nucleoside triphosphates (NTPs), deoxy-NTPs (dNTPs), and dideoxy-NTPs were from Pharmacia. Sephadex G-25_f and phosphocellulose P-11 were purchased from Pharmacia and Whatman, respectively. Restriction enzymes and other DNA-modifying enzymes were from New England Nuclear or Gibco BRL and were used according to the manufacturers' instructions. [α -³²P]NTPs and [α -³²P]dNTPs (3,000 Ci/mmol) were purchased from DuPont, NEN. Synthetic dinucleotides (ApA, CpA, CpC, UpC, and CpU) were purchased from Sigma.

Media and strains. Yeast media were as described by Sherman et al. (44). The genotype of yeast strain SA23-1A is *MAT α rel1::HIS3 trp1 leu2 ura3-1 met4-1o lys2-1o ade2-1o leu2 his3- Δ 200 can1-100o cyh2/pSA23 (CEN6 ARSH4 LEU2 CYH2 RET1)*, and that of strain GDS 1-25A is *MAT α trp1 leu1 ura3-1 met4-1o lys2-1o ade2-1o leu2 can1-100o. E. coli* TG1 [K-12 Δ (*lac-pro*) *supE thi hsd D5/F traD36 proA⁺ B⁺ lacI^q lacZ Δ M15*] was used for all procedures involving M13 and DH5 α [*F⁻ Δ (lacZYA-argF)U169 endA1 recA1 hsdR17(r_K⁻ m_K⁺) deoR [ϕ 80dlac Δ (lacZ)M15] thi-1 supE Δ 44 λ -gyrA96 relA1]* was used for plasmid DNA preparation.

In vitro mutagenesis. The *SUP4* gene was cloned as a 266-bp *Bam*HI fragment from the plasmid pAsup4-4 (2) into the *Bam*HI site of M13mp18. The *Bam*HI fragment, which contains *SUP4* along with 103 bp of 5' flanking DNA and 55 bp of 3'-flanking DNA, contains all of the *cis*-acting elements necessary for maximal *in vivo* ochre suppression. Single-strand DNA was prepared from M13mp18::*SUP4* by standard methods, and site-directed mutagenesis of the *SUP4* transcription start site region was performed with a Sculptor mutagenesis kit from Amersham. The *SUP4* start site mutants produced are listed in Table 1. The mutagenic oligonucleotides were prepared by the Howard Hughes Medical Institute chemical synthesis facility at the University of Washington. The presence of the desired mutation(s) was confirmed by dideoxy sequencing.

Chromosomal integration of *SUP4* start site mutants. Plasmid YIp5::*SUP4*-U(IV) contains a 4.5-kb segment of yeast chromosome X, including the *SUP4* locus, as an *Eco*RI-*Hind*III fragment (17) in the yeast-integrating vector YIp5 (45). The isolated 266-bp *Bam*HI fragments from each of the M13mp18::*SUP4* start site mutants were individually ligated into the ~6.7-kb *Bam*HI fragment from YIp5::*SUP4*-U(IV). The 266-bp fragments replace an ~3.3-kb fragment containing the entire *SUP4*-U(IV) allele and a portion of the YIp5-encoded *tet^R* gene (see Fig. 1). Restriction analysis of plasmid minipreps was performed to verify the nature of the recombinant DNAs isolated from ampicillin-resistant transformants. Plasmids with *Bam*HI restriction patterns of 6.7 and 0.27 kb were further analyzed by restriction digestion with *Sma*I, which cuts once in YIp5 and once in the *SUP4* gene, to determine the orientation and copy number of the insert. Single-insert plasmids with the *SUP4* mutant start sites distal to the *tet^R* gene (Fig. 1) were chosen. One microgram of plasmid DNA was digested to completion with *Bst*EII, which cuts once in the yeast 5'-flanking DNA. The linear DNA was transformed into yeast strain SA23-1A by the lithium acetate method (41), and stable transformants were selected on complete minimal media lacking uracil. Chromosomal DNAs were prepared from several URA⁺ transformants for each mutant (44), and the integration of a single plasmid at the *SUP4* locus was confirmed by electrophoresis and Southern blot analysis of 10 μ g of chromosomal DNA digested with *Pvu*II, which cuts once in the YIp5 portions of the YIp5::*SUP4* plasmids. The blots were probed with a 920-bp *Bam*HI-*Bgl*II fragment from the *SUP4* 5'-flanking region of YIp5::*SUP4*-U(IV) which had been labeled with [α -³²P]dATP with a Random Primed DNA labeling kit from Boehringer Mannheim. With this probe, Southern hybridization should detect *Pvu*II fragments of approximately 12 kb in DNA from cells lacking an integrated YIp5::*SUP4*, two bands of approximately 8.5 and 13 kb with a single integrant, and bands of 7.0, 8.5, and 13 kb in the case of multiple integration.

Screening *SUP4* start site mutants for decreased ochre suppression. Cells with a single integrated copy of a *SUP4* start site mutant were patched with sterile toothpicks from the transformation plate lacking uracil onto new plates lacking uracil and grown at 30°C for 3 to 5 days. Cells were replica plated onto different types of media to screen for ochre suppression of the SA23-1A biosynthetic auxotrophies in the following order: three consecutive yeast extract-peptone-dextrose (YEPD) plates, followed by single plates containing complete minimal medium lacking either adenine, lysine, or methionine and another YEPD plate. After growth for 5 days at 30°C, the plates were scored for color yield (YEPD) and cell growth (dropouts).

In vitro transcription reactions. In vitro transcription extracts (S-100) were prepared from strain GDS 1-25A essentially as described by Koski et al. (23). Modifications to the original procedure and subsequent phosphocellulose (P-11) chromatography were as described by Shaaban et al. (42).

All transcription reactions were performed with a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.9), 7 mM MgCl₂, and 5 mM dithiothreitol.

Multiple-round transcription reactions were performed in a final volume of 20 μ l and contained 10 fmol of YIP5 DNA containing either authentic *SUP4* or one

of the *SUP4* start site mutants and 0.5 mM each ATP, CTP, and UTP, 0.05 mM cold GTP, and 5 μ Ci of [α -³²P]GTP. The final KCl concentration was adjusted to 150 mM to promote efficient processing of the primary transcription product or to 70 mM to inhibit processing. The reactions were initiated by adding 2 to 4 μ l of transcription extract and were terminated after 30 min at 25°C by addition of the same volume of stop mixture to give final concentrations of 1% sodium dodecyl sulfate, 0.4 mg of proteinase K per ml, 5 mM EDTA, and 0.2 mg of yeast total RNA per ml, and then the reaction mixture was treated at 65°C for 20 min. S-100 extract was used to determine RNA processing activity, and P-11 extract was used for all other reactions. Recovery and electrophoresis on 15% polyacrylamide gels (29:1) was as previously described (2). Variations to the standard assay conditions are described in the text.

For single-initiation-event kinetic assays, preinitiation complexes were formed in a total volume of 10 μ l by mixing 10 fmol of DNA and 2 to 4 μ l of P-11 extract under buffer conditions as described above with 150 mM KCl for 30 min at 25°C. In some cases, the initiating NTP was included in the preinitiation complex mixture at a concentration of 0.5 mM. Transcription was initiated by addition of the same volume of a chase solution, containing buffered KCl as in the preinitiation complex, to give final concentrations of 0.5 mM ATP and CTP, 1 μ M cold UTP, and 10 μ Ci of [α -³²P]UTP for UTP labeling or 0.5 mM ATP and UTP, 1 μ M cold CTP, and 10 μ Ci of [α -³²P]CTP for CTP labeling. Under these conditions, elongating polymerases became arrested at the point of incorporation of the first G, forming a stable ternary complex containing a 17-nucleotide-long transcript on *SUP4*. The reactions were stopped at various times as indicated in the text and processed as described above.

For heparin sensitivity assays, preinitiation complexes were formed either as described above for the kinetic assays or in the presence of subsets of NTPs, at 500 μ M each, as described in the text. Equal volumes of chase solution were added to the preformed complexes so that the final concentrations were 0.5 mM each ATP, CTP, and UTP, 1 μ M cold GTP, 5 μ Ci [α -³²P]GTP, and 0.5 mg of heparin per ml. After 10 min at 25°C, the reactions were stopped and processed as described above.

For analysis of abortive initiation products, the reactions were performed in a final volume of 5 μ l. DNA, cold NTPs, and synthetic initiator dinucleotides were combined in a total volume of 3.4 μ l and the reactions were initiated by adding 1.6 μ l of a 3 $\times$ buffer solution containing extract and radioactivity so that the final concentrations were 100 mM KCl, 2.5 fmol of DNA, 5 μ Ci of [α -³²P]CTP (when *SUP4* was used as the template) or [α -³²P]UTP (when *SUP4*-CTC2 [CTCCTC] was used as the template), and 0.5 μ l of P-11 extract per reaction mixture. Concentrations of cold NTPs and synthetic initiator dinucleotides used in individual reaction mixtures are described in the text and figure legends. After 30 min at 25°C, the reactions were terminated by addition of 4 μ l of 95% formamide-20 mM EDTA-0.05% bromophenol blue-0.1% xylene cyanol FF and heating at 100°C for 10 min. Products were run on 28% polyacrylamide gels (25:3) until the bromophenol blue was approximately two-thirds through the gel. Following electrophoresis, the undried gels were left on one plate, covered with plastic wrap, and exposed to X-ray film at -70°C.

RESULTS

Sequence changes near the transcription start site of the *SUP4* gene. The sequence element CAACAA occurs in the nontranscribed strand of DNA at or near the transcription start site of many yeast tRNA genes (10, 19, 39), and the start sites have been mapped to the first A in several cases (21). To investigate its role in gene transcription, we made base substitution mutations within the CAACAA element of the yeast *SUP4* tRNA^{Tyr} gene. Wild-type and mutant *SUP4* gene sequences are shown in Table 1, with capital and lowercase letters denoting base positions relative to the transcribed and upstream regions of the parental *SUP4* gene. Differences from the wild-type sequence are underlined and in boldface type. The mutant alleles were integrated at the *SUP4* locus of an *S. cerevisiae* strain marked with the ochre-suppressible alleles *ade2-1*, *lys4-1*, and *met4-1* (Fig. 1). The different integrant strains were scored for their ability to suppress the marker alleles by replica plating onto appropriate media. The levels of *SUP4* tRNA needed to suppress the mutant alleles differ in the order *ade2-1* > *lys4-1* > *met4-1*. Unsuppressed *ade2-1* cells are dark red, fully suppressed cells are beige to white, and partially suppressed cells are pink in color to various degrees (17, 24, 42).

Different sequences near the *SUP4* transcription start site produced different levels of suppression, as summarized in Table 1. All of the single-base-pair substitutions at positions

FIG. 1. Plasmid constructs and integration scheme for *SUP4* start site mutants. Start site mutants were isolated as 266-bp *Bam*HI fragments, which contain all of the sequence information necessary for in vivo *SUP4* expression. The fragments were used to replace an ~3.3-kb *Bam*HI fragment from a vector containing 4.5 kb of DNA from the *SUP4* region of yeast chromosome X. The orientations of the inserts were determined by *Sma*I restriction analysis. Plasmids with a *SUP4* mutant in the same orientation as that of the original *SUP4-o* (i.e., transcription of the *SUP4* gene oriented towards the tetracycline resistance gene) were linearized at the unique *Bst*EII site and integrated at the *sup4*⁺ locus of the chromosome. Integration of a single Ylp5::*SUP4* plasmid was monitored by selection for a Ura⁺ phenotype and by Southern analysis as described in Materials and Methods.

TABLE 1. In vivo suppression phenotypes of mutant *SUP4* alleles

<i>SUP4</i> allele or vector	Sequence ^b	Suppression phenotype on ^c :				Suppression index ^d
		YEPD	-Ade	-Lys	-Met	
<i>SUP4-o</i>	ctctttcttcAACAATTAAA	W	+++	+++	+++	7
<i>SUP4-C₋₁A</i>	ctctttcttc a AACAATTAAA	W	+++	+++	+++	6.5
<i>SUP4-C₋₁G</i>	ctctttcttc g AACAATTAAA	W	+++	+++	+++	6.5
<i>SUP4-C₋₁T</i>	ctctttcttc t AACAATTAAA	W	+++	+++	+++	7
<i>SUP4-A₊₁G</i>	ctctttcttc G ACAATTAAA	W	+++	+++	+++	7
<i>SUP4-A₊₁C</i>	ctctttcttc C ACAATTAAA	W	+++	+++	+++	7
<i>SUP4-A₊₁T</i>	ctctttcttc T ACAATTAAA	W	+++	+++	+++	7
<i>SUP4-CGC1</i>	ctctttcttc G CCAATTAAA	W	+++	+++	+++	7
<i>SUP4-CGC2</i>	ctctttcttc G CC G CTTAAA	LP	+	+++	+++	5.5
<i>SUP4-CTC1</i>	ctctttcttc T CCAATTAAA	R	-	+/-	+++	1.5
<i>SUP4-CTC2</i>	ctctttcttc T C C TCTTAAA	R	-	-	+++	1
<i>SUP4-CTC3</i>	ctctttcttcA A C T CTTAAA	W	+++	+++	+++	7
<i>SUP4-US1</i>	ctc aa tcttcTCCTCTTAAA	R	-	+/-	+++	1.5
<i>SUP4-US2</i>	ctctttc aa cTCCTCTTAAA	R	-	+/-	+++	1.5
YCp50	None	R	-	-	-	0

^a The *SUP4* start site mutants are named according to sequence changes they contain. YCp50 contains no *SUP4* gene.

^b Sequence changes, shown for the nontranscribed DNA strand, in the vicinity of the transcription initiation region are underlined and in boldface type. Uppercase letters represent sequence positions which are transcribed in *SUP4-o*; lowercase letters refer to positions upstream of the transcription initiation site of *SUP4-o*.

^c The suppression phenotypes are the pertinent growth characteristics of the mutants on various indicator media. The colors formed on rich medium (YEPA) are indicated with W for white, LP for light pink, and R for red. To indicate growth on complete media lacking defined nutrients, - signifies no growth, + signifies poor growth, and +++ signifies robust growth. -Ade, medium without adenine; -Lys, medium without lysine; -Met, medium without methionine.

^d The suppression index was determined by assigning a numerical value to the phenotypes observed. On YEPA, red is 0, dark pink is 1, pink is 2, light pink is 3, and white is 4. On each dropout plate, +++ is 1.

FIG. 2. Characterization of *SUP4* start site mutants producing altered suppression phenotypes. A master plate was replica plated to the plates indicated in the figure to score for suppression of ochre-suppressible nonsense mutations by different alleles of *SUP4*. The color yield on YEPD provides a sensitive measure of the amount of suppression of the *ade2-1* biosynthetic defect. Cells bearing an unaltered *SUP4-o* allele are beige or white, and this is taken as 100% suppression, while those containing YCp50 (no *SUP4* gene) are dark red, indicating no suppression. Intermediate suppression levels can generate many readily distinguishable shades of pink (see the text and related references). Growth on complete minimal media lacking adenine (–ADE), lysine (–LYS), or methionine (–MET) indicates suppression of *ade2-1*, *lys2-1*, and *met4-1*, respectively.

C_{–1} and A_{–1} and both the CGC1 and CGC2 *SUP4* alleles gave high levels of in vivo ochre suppression. Sequence changes that extended the upstream pyrimidine-rich sequence into the transcribed region had significant deleterious effects on the levels of suppression. Figure 2 shows the growth phenotypes associated with the strongest of the *SUP4* mutant alleles. Both the *SUP4*-CTC1 (CTCCAA) and *SUP4*-CTC2 (CTCCTC) mutants were dark red on YEPD plates, indicating little or no adenine suppression. The cells were Met⁺, indicating that both *SUP4*-CTC1 and *SUP4*-CTC2 produce low but detectable levels of suppressor tRNA. However, the *SUP4*-CTC2 allele was unable to suppress either the lysine or the adenine defect while *SUP4*-CTC1 grew poorly in the absence of lysine and did not suppress *ade2-1* (Table 1 and Fig. 2). The *SUP4*-CTC3 allele, CAACTC, provided wild-type levels of suppression (not shown). The level of suppression corresponding to different *SUP4* start site sequences can be summarized as follows:

To determine the basis for the effects of AA-to-TC substitutions upon *SUP4* expression, comparative assays specific for several aspects of RNA polymerase III transcription were carried out with *SUP4* genes with wild-type and mutant start site sequences. For most of the in vitro assays, we used *SUP4*-CTC2, the mutant allele with the most strongly affected in vivo phenotype. Other alleles were used where appropriate.

Multiple-round in vitro transcription of *SUP4* and *SUP4*-CTC2. Several aspects of *SUP4*-CTC2 in vitro transcription were unchanged compared with that of *SUP4*. Under standard

conditions in an S-100 extract (see Materials and Methods) the level of tRNA^{Tyr} transcribed from *SUP4*-CTC2 was comparable to that transcribed from *SUP4*. Both RNAs were accurately processed, giving transcripts of ~107 (pre-tRNA^{Tyr}), 89 (mature 5' and 3' ends), and 75 (mature tRNA) nucleotides, confirming our expectation that the defect in suppression noted for *SUP4*-CTC2 is not due to a lack of proper RNA processing.

Under similar conditions with a P-11 polymerase III transcription extract, varying the DNA template concentration from 25 to 800 pM showed that saturation was reached at around 300 pM, at which point the final yields of in vitro-synthesized RNA were the same with both templates. In addition, the slopes in the linear range (between 0 and 300 pM) of the saturation curves were identical for both templates, indicating that transcription complexes were assembled with equal avidity on each of the DNAs (not shown).

To assess the ability of each template to compete for transcription components in the presence of a second RNA polymerase III promoter, saturating amounts (500 pM) of either *SUP4* or *SUP4*-CTC2 were incubated with P-11 extract and NTPs in the presence of increasing amounts of a plasmid bearing a yeast tRNA₃^{Leu} gene that is efficiently transcribed by RNA polymerase III. The presence of the tRNA₃^{Leu} gene reduced the level of transcription of each *SUP4* template by the same extent, the reduction being twofold with 200 pM of tRNA₃^{Leu} added (not shown). These results indicate that *SUP4* and *SUP4*-CTC2 are similar in their abilities to sequester limiting transcription components in the presence of a strongly competing polymerase III promoter.

NTP requirements for *SUP4* and *SUP4*-CTC2 in vitro transcription. Since placing as few as two pyrimidines at the transcription start site caused diminished *SUP4* gene expression, it seemed possible that the *SUP4*-CTC1 and -CTC2 defects were due to an unusually high *K_m* for pyrimidines at initiation. The requirements for individual nucleotides were tested on *SUP4* and *SUP4*-CTC2 with P-11 cell extract by labeling with 50 μM [α -³²P]GTP, which is not required until template position 17 of each allele. Two of the first three nucleotides were maintained at high levels (500 μM), and the concentration of the third varied. The largest difference between the two templates was seen when the concentration of CTP was varied; *SUP4*-CTC2 required a fivefold greater concentration of CTP to reach 50% saturation than did *SUP4*. The large difference in half-saturating levels of CTP was not caused by an unusually high requirement for CTP during transcription of *SUP4*-CTC2 but was due to the very low levels of CTP required for transcription of *SUP4*; half-maximal transcription was reached at less than 10 μM CTP for *SUP4*, but about 50 μM CTP was required to achieve similar levels with *SUP4*-CTC2 (Fig. 3A). By contrast, UTP requirements were similar with both templates (apparent *K_m*, 30 μM for each) and *SUP4* required approximately twice as much ATP as *SUP4*-CTC2 (about 30 and 60 μM, respectively) to attain 50% of the maximal transcription level (not shown).

Under the standard transcription conditions, the rates of pre-tRNA^{Tyr} accumulation were the same for both *SUP4* and *SUP4*-CTC2 (not shown). Significant differences were observed either when the concentrations of all four ribonucleoside triphosphates were lowered to 50 μM or when that of CTP alone was lowered; in both cases, the amount of *SUP4*-CTC2 RNA produced in a 30-min reaction was only about 25 to 30% of the maximal *SUP4* value (Fig. 3B and C). Transcription kinetics for both templates were identical at a low concentration of UTP, while a low concentration of ATP caused a slight reduction in *SUP4* transcript yield compared with that of *SUP4*-CTC2.

FIG. 3. Influence of CTP on multiple-round transcription by *SUP4* and *SUP4-CTC2*. (A) Saturation curves for CTP on both templates. The concentrations of ATP, UTP, and [α -³²P]GTP were constant at 500, 500, and 50 μ M, respectively, and the concentrations of CTP were varied to between 10 and 500 μ M. (B) Kinetics of pre-tRNA^{Tyr} formation in the presence of 50 μ M each ATP, CTP, UTP, and [α -³²P]GTP. (C) Kinetics of pre-tRNA^{Tyr} formation in the presence of 500 μ M each ATP and UTP and of 50 μ M CTP and [α -³²P]GTP.

Kinetics of productive transcription initiation. To further characterize the transcription defect of *SUP4-CTC2* and to study its unusual [CTP] dependence, we carried out single-cycle-transcription experiments in the absence of GTP. Under these conditions, polymerase III transcripts from both the *SUP4* and *SUP4-CTC2* templates became stalled immediately before the first transcribed G. Because neither these stalled transcripts nor polymerase III was released from the template, reinitiation could not occur and the levels of transcript produced provided a measure of the number of polymerase III molecules that formed stable elongation complexes (21, 42). The sizes of the *SUP4* (17 nucleotides) and *SUP4-CTC2* (16 nucleotides) templates differed slightly because the start sites occurred at different points (see below).

While equal levels of product were formed in 30-min reactions on the two templates, the initial rate of product accumulation was approximately six times faster for *SUP4* than for *SUP4-CTC2* (Fig. 4A). This rate difference persisted even when the initial dinucleotide was formed during a 30-min preincubation with ATP (*SUP4*) or CTP (*SUP4-CTC2*) prior to the addition of [α -³²P]UTP and the third NTP (Fig. 4B). Therefore, the rate-limiting step which slowed the formation of stable elongation complexes on the mutant template occurred after formation of the first phosphodiester bond. Conceivably, this differential effect could be attributed to the initial occurrence of UMP, the limiting nucleotide, at position 3 in the *SUP4-CTC2* RNA, versus position 6 in the *SUP4* transcript. Two lines of evidence argue against this. *SUP4-CTC1* (CTCC AA) RNA also initiates at C₂ (not shown), and UTP is not required until residue 5 (equivalent to residue 6 of *SUP4*). In limiting UTP, the kinetics of stable complex formation on CTC1 were also diminished relative to those of *SUP4* (Fig. 4C), indicating that U at position 3 does not constitute the sole detrimental effect on *SUP4-CTC2* transcription. When *SUP4* transcription was performed in low-concentration CTP (required at position 3 of the nascent transcript) instead of low-concentration UTP, transcription kinetics were relatively unaffected (Fig. 4C).

These results indicate that the efficiency of stable complex formation can be modulated by the base composition at the 5' ends of the newly formed transcripts. The observations that transcription rates were not substantially elevated for either

SUP4-CTC1 or *SUP4-CTC2* containing preformed pppCpC suggest that transcripts initiated with consecutive C's are not optimal substrates for chain extension.

Transcript length differences between *SUP4* and start site mutants. The electrophoretic mobility of the *SUP4-CTC2* transcript suggests that it is 1 nucleotide shorter than the *SUP4* transcript, which starts at the first A of CAACAA. This observation implies that the initial two nucleotides of the *SUP4-CTC2* transcript are those underlined in CUCCUC, an assignment fully consistent with the strong dependence of *SUP4-CTC2* upon [CTP]. To confirm this start site, we used dinucleotide priming and electrophoretic size determination with denaturing polyacrylamide gels. This methodology has previously been applied to map several polymerase III transcript start sites, including *SUP4* (21). When *SUP4-CTC2* transcription was primed with CpC, which corresponds to the RNA encoded by the +2 and +3 positions of the template, the product comigrated with the unprimed, phosphatase-treated transcript, establishing that their lengths were the same. Transcripts generated by priming *SUP4-CTC2* transcription with UpC (+1 and +2) or CpU (−1 and +1) were 1 and 2 nucleotides longer, respectively, than the phosphatase-treated unprimed transcript (data not shown). All transcript lengths were fully consistent with our expectations from the *SUP4-CTC2* DNA sequence. Transcription of the +1 pyrimidine variants CC₊₁ACC and CT₊₁CAA also produced 16-mer RNAs, indicating that transcription started at A₊₂ (data not shown).

SUP4-CTC2 transcription in 50 μ M [α -³²P]CTP generated approximately equal yields of 16-mer and 17-mer transcripts when UTP was at 500 μ M and 16-mer transcripts alone when UTP was at 50 μ M (not shown). Together, these results suggest that within a small window, a purine, ATP, is preferred over pyrimidines as an initiator nucleotide and that initiating UTP, or newly formed transcripts containing 5'-terminal uridine residues, may be highly unstable at the catalytic center because of weak interactions with either DNA or protein. The extremely weak hydrogen bonds formed by rU::dA base pairs (31) may influence the ability of polymerase III to initiate with U, though U₊₁ start sites were not more frequent in the presence of 5-bromo-UTP, which pairs more strongly with A (not shown).

FIG. 4. Single-round transcription kinetics. Binary preinitiation complexes or complexes preincubated with NTPs sufficient to form the first phosphodiester bond were formed on *SUP4* and *SUP4-CTC2*. Single-round transcription was initiated by the addition of a cocktail of NTPs, so that the final concentrations in each reaction mixture were 500 μ M for both ATP and CTP and 1 μ M for [α - 32 P]UTP. Samples were withdrawn, and the reactions were stopped at various times. (A) Autoradiogram showing transcription kinetics from preformed preinitiation complexes. (B) Autoradiogram showing transcription kinetics from complexes incubated with NTPs to form the first phosphodiester bonds: pppApA for *SUP4* and pppCpC for *SUP4-CTC2*. For panels A and B, the times of reaction are indicated above the gels. Lanes labeled C are control reactions performed for 30 min. (C) Plot of product accumulation versus time for *SUP4* transcription after labeling with 1 μ M [α - 32 P]UTP (filled squares) or 1 μ M [α - 32 P]CTP (filled triangles) and for transcription of *SUP4-CTC1* and *CTC2* in 1 μ M [α - 32 P]UTP.

Transcript slippage on *SUP4-CTC2*. A second surprising feature was revealed by transcription experiments in the absence of GTP. At low concentrations of ATP (less than 50 μ M), two major transcripts were produced from the *SUP4-CTC2* template, one with the mobility of the 16-mer transcript produced under conditions of high concentrations of ATP and the second with the mobility of a 19-mer transcript (Fig. 5). Lowering the concentration of ATP resulted in an increase in the relative amount of the 19-mer, and the ratio of 19-mer to 16-mer was found to increase with time when *SUP4-CTC2* preinitiation complexes were incubated for various times with CTP plus [α - 32 P]UTP and then chased with 500 μ M ATP (not shown). A second transcript was not observed from the *SUP4* template under conditions of low concentrations of ATP. ATP is not required until position 7 of the *SUP4-CTC2* RNA, at which point three consecutive A's are encoded, while A is present at 7 of the first 10 positions of *SUP4* RNA (5'-terminal transcript sequences are 5'-pppCCUCUAAAA for *SUP4-CTC2* and 5'-pppAACAAUAAAA for *SUP4*). The 19-nucleotide transcript was not seen when *SUP4-CTC1* (CTCCAA) was transcribed under conditions of limiting ATP (not shown), indicating that transcribed RNA sequences present in the *CTC2* gene but not in the *CTC1* gene were essential for the formation of the 19-nucleotide transcript (Table 1). These results suggest that, in conditions of limiting ATP, transcrip-

tion complexes pause at position +6, just before the AAA sequence of *SUP4-CTC2*. The all-pyrimidine hexanucleotide formed prior to the pause can be repositioned 3 nucleotides upstream on the template, and the newly positioned 3' end can then be extended by templated nucleotide addition (Fig. 6). Following transcript slippage, as shown in Fig. 6, the newly positioned RNA contains an unpaired 5' end and a non-Watson-Crick G-U base pair. Transcript slippage to produce

FIG. 5. Transcript-length heterogeneity from *SUP4-CTC2* in a low concentration of ATP. Transcription was performed in the absence of GTP with 500 μ M CTP and various amounts of ATP and by labeling with 1 μ M [α - 32 P]UTP. Lanes 1, 3, 5, and 7, *SUP4* transcription at 10, 20, 50, and 100 μ M ATP, respectively; lanes 2, 4, 6, and 8, *SUP4-CTC2* transcription at 10, 20, 50, and 100 μ M ATP, respectively.

FIG. 6. Transcript slippage on *SUP4*-CTC2. Transcription in the presence of limiting concentrations of ATP generates complexes which are stalled at the site of addition of the first adenylate and contain the oligoribonucleotide pppCCU CUU. The paused product shifts 3 nucleotides upstream, and the 3' end acts as a primer for continued transcription.

noncomplementarity between the 5'-terminal ribonucleotide and the DNA template has been proposed to explain the origins of separate but related transcripts produced from genes at the S1 nuclease site in *ColE1* DNA (35). As was suggested for the *ColE1* case, the *SUP4*-CTC2 data indicate that conditions which promote RNA polymerase pausing during early transcription may increase the likelihood of abortive release and/or repositioning of the short nascent RNA.

Sensitivity to heparin. The transcript repositioning results suggest that short RNAs made on the *SUP4* but not on the *SUP4*-CTC2 template act to stabilize the transcription complex. To further probe initiation and early elongation complex stability, we measured the ability of *SUP4* and *SUP4*-CTC2 transcription complexes to make complete transcripts when challenged by heparin at various early stages. In the presence of heparin, stable ternary complexes are able to continue transcript elongation but cannot reinitiate transcription (21). Heparin either was added to preformed complexes directly and then chased with all four NTPs (providing measurements of the levels of stability of existing complexes) or was added to complexes together with NTPs (establishing a competition between stabilization by RNA synthesis and inactivation by heparin).

Both the *SUP4* 17-mer and the *SUP4*-CTC2 16-mer complexes, formed by transcription in the absence of GTP, showed complete resistance to heparin, since 100% of the stalled complexes could be chased to full length when challenged with heparin by either method (not shown). Thus, at this transcript length, the transcription complexes have progressed to stable ternary complexes. To examine the intermediates leading up to this stable condition, we performed similar experiments with complexes with no RNA, with a preformed RNA dinucleotide (pppApA for *SUP4* and pppCpC for *SUP4*-CTC2), and with either the A + C product on *SUP4* (pppAACAA) or the C + U product on *SUP4*-CTC2 (pppCCUCCUU).

Upon direct challenge by heparin (prior to NTP addition), there was little difference in levels of heparin sensitivity between complexes formed on *SUP4* and similar complexes formed on *SUP4*-CTC2. Binary complexes (no RNA) and dinucleotide-containing complexes were completely and nearly completely inactivated, respectively. However, complexes containing the 5- and 6-nucleotide-long RNAs were nearly completely resistant to heparin, yielding values more than 75% of the 17-mer or 16-mer control values (not shown).

When preformed complexes were treated with a mixture of heparin and NTPs, heparin resistance was dependent on the composition of the 5' ends of the RNAs. Under these conditions, *SUP4* binary complexes (no RNA) were substantially resistant to the effects of heparin; the final yield of full-length

FIG. 7. Effect of heparin on transcription from *SUP4* and *SUP4*-CTC2. The bar graph in the upper portion of the figure shows the percentages of full-length pre-tRNA^{Tyr} produced from preformed complexes in the presence of heparin. The percentages given for complexes formed in either the absence of nucleotides or the presence of only the NTP encoding the first dinucleotide (pppApA from ATP on *SUP4* and pppCpC from CTP on *SUP4*-CTC2) are relative to complexes formed in the presence of ATP, CTP, and UTP on each template. The timeline in the lower portion of the figure is a schematic for the experimental protocol; template DNA, P-11 enzymes, and NTPs, as indicated, were preincubated prior to the addition of a chase solution to make the final reagent concentrations 0.5 mg of heparin per ml, 0.5 mM each ATP (A), CTP (C), and UTP (U), and 1 μ M [α -³²P]GTP.

tRNA^{Tyr} was about 50% of that produced from a preformed 17-mer complex (Fig. 7). Conversely, *SUP4*-CTC2 binary complexes were far more sensitive; the final yield of full-length transcript was only about 10% of that generated by a preformed 16-mer complex. The *SUP4* dinucleotide-containing complexes gave even more heparin resistance than the binary complex; the yield of full-length product produced from *SUP4* plus ATP was about 80% of that produced from a preformed 17-mer. By contrast, product yield from the *SUP4*-CTC2 plus CTP reaction represented only 23% of the 16-mer control (Fig. 7). As seen with heparin treatment without NTPs, ternary complexes with the 5- and 6-mer RNAs were almost completely heparin resistant (not shown).

These results show that RNA chain length is an important factor in the formation of stable complexes and that, even for transcripts composed entirely of pyrimidines, a relatively short RNA can induce stabilization. However, dinucleotides alone in a stalled complex do not cause stable complex formation, suggesting that formation of the first phosphodiester bond is not sufficient to promote a stable, elongation-competent state. The differences noted when heparin and NTPs were added together to transcription complexes show that during the early stages of transcription there is a competition between inactivation by heparin and complex stabilization engendered by transcript

FIG. 8. Abortive initiation from *SUP4-CTC2*. Abortive product formation with CpC priming from a cognate template and noncognate templates. The reaction mixtures contained 5 μ Ci [α - 32 P]UTP and 1 μ M cold UTP. Dinucleotides, when present, were at 500 μ M. Additional components are given in the lane descriptions noted below and listed above the autoradiogram. Reactions were stopped with formamide and loaded directly onto a denaturing polyacrylamide gel. Lane 1, [α - 32 P]UTP (U) alone; lane 2, CpC; lane 3, *SUP4*; lane 4, *SUP4-CTC2*; lane 5, *SUP4* plus 500 μ M each ATP (A) and CTP (C); lane 6, *SUP4-CTC2* plus 500 μ M each ATP and CTP; lane 7, Ylp5 vector DNA (no *SUP4*) plus CpC; lane 8, *SUP4* plus CpC; lane 9, *SUP4-CTC2* plus CpC; lane 10, *SUP4* plus CpC plus 10 μ M CTP; lane 11, *SUP4-CTC2* plus CpC plus 10 μ M CTP; lane 12, *SUP4* plus CpC plus 500 μ M ATP; lane 13, *SUP4-CTC2* plus CpC plus 500 μ M ATP; lane 14, *SUP4* plus CpC plus 10 μ M CTP plus 500 μ M ATP; lane 15, *SUP4-CTC2* plus CpC plus 10 μ M CTP plus 500 μ M ATP. Formation of CpCpU on the transcribed strand of *SUP4-CTC2* is indicated below the autoradiogram.

initiation and/or elongation. The results suggest that RNAs consisting of the first few nucleotides transcribed from *SUP4-CTC2* are less likely to be extended than those transcribed from *SUP4*, rendering the CTC2 complexes unstable and sensitive to attack by heparin.

Abortive initiation on *SUP4* and *SUP4-CTC2*. The results just described suggest that short nascent RNAs formed by RNA polymerase III on *SUP4-CTC2* do not contribute to the formation of a stable ternary transcription complex to the same extent as the short RNAs formed on *SUP4*. One possible explanation for this observation may be that the short, oligopyrimidine nascent RNAs formed on *SUP4-CTC2* are themselves less stably bound in the ternary complex than the corresponding purine-rich RNAs formed on *SUP4*. In order to directly address this possibility, we tested for abortive product synthesis from *SUP4* and *SUP4-CTC2*.

For the gel autoradiogram shown in Fig. 8, each reaction contained transcription extract and [α - 32 P]UTP. The presence of template DNA, a synthetic dinucleotide primer, CpC, and additional unlabeled NTPs in certain reactions are indicated above the lanes in Fig. 8 and in the figure legend. When the *SUP4-CTC2* template was incubated with transcription extract, CpC, and [α - 32 P]UTP, an apparent abortive trinucleotide product, CpC 32 pU, was produced (Fig. 8, lane 9). This identification is based on the following observations. The formation of this product required the presence of both the CpC dinucleotide primer and *SUP4-CTC2* (Fig. 8, lanes 1, 2, 4, and 9). Replacement of the *SUP4-CTC2* template by the Ylp5 vector

alone or the vector containing authentic *SUP4* resulted in a greatly diminished yield of the labeled product (Fig. 8, lanes 7 and 8). When CTP or CTP and ATP together were included in the reaction with CpC and *SUP4-CTC2*, the yield of abortive products was substantially reduced (Fig. 8, lanes 11 and 15). However, the addition of ATP alone, which is not required until position 7 of the nascent *SUP4-CTC2* RNA, did not alter the yield (Fig. 8, lane 13).

In addition to the CpC extension reaction just described, the dinucleotide UpC can be specifically extended with [α - 32 P]CTP in the presence of *SUP4-CTC2* but not Ylp5 or authentic *SUP4* (not shown). UpC primes RNA synthesis 1 nucleotide upstream of CpC on the *SUP4-CTC2* template strand, as shown below (boldface type indicates the first added nucleotide, which is radioactive).

Formation of UpCpC was strongly inhibited in the presence of the next encoded nucleotide, UTP, but was unchanged in the presence of ATP.

From these results, we infer that RNA polymerase III is able to repetitively synthesize and release the trinucleotides CpCpU and UpCpC from the *SUP4-CTC2* template and that the loss of the trinucleotide signal observed in the presence of the next-encoded nucleotide is due to a transition of the initially transcribing complex from an unstable abortive state to a more stable form following template-dependent synthesis of RNAs greater than or equal to 4 nucleotides in length.

A similar strategy was used to test for abortive product formation from *SUP4*. Synthesis was primed with the dinucleotide ApA in the presence of [α - 32 P]CTP, which is encoded at position 3 of the *SUP4* gene. As stated earlier, ApA is effective at specifically priming 17-mer synthesis from *SUP4* in the reaction mixture lacking GTP. Primed synthesis from *SUP4* with ApA and labeled CTP generated an apparent trinucleotide product at a yield much lower than that seen from the CpC reaction with *SUP4-CTC2*. However, product formation with ApA, though dinucleotide dependent, was not template dependent and the product was formed at approximately equal yields in the presence of either Ylp5 (no *SUP4* gene) or *SUP4-CTC2* or in the absence of DNA, indicating that it does not represent an abortive initiation product (results not shown). Despite the minor labeling of ApA in a template-independent fashion, it is clear that abortive synthesis of short products is much more highly favored on *SUP4-CTC2* than on *SUP4*.

DISCUSSION

Transcripts initiated by purine nucleotides are favored. Our experiments with in vivo and in vitro transcription of mutant yeast *SUP4* genes show that sequence changes in the transcription initiation region can alter the level of transcripts produced. The sequence element CAACAA is not absolutely required for high levels of transcription in vivo, as many *SUP4* start site mutants show wild-type or near wild-type levels of in vivo suppression. Of the changes in the 13 mutant sequences analyzed, only the two base substitutions which place consecutive pyrimidines within the window utilized by RNA polymerase III for transcription initiation had a substantial deleterious effect on suppression levels. These mutant alleles, CTC1 and CTC2, share an AA-to-TC mutation at the +1 and +2 positions.

The window for transcription initiation. Our results confirm and extend the findings of Fruscoloni et al. (11), who investi-

gated in vitro transcription using start site variants of the yeast $tRNA_{3'}^{\text{Leu}}$ gene. The ability of RNA polymerase III to scan the *SUP4* initiation region for a preferred start site residue is limited. Transcription start sites were not seen at adenosines at positions 4 and 5 of *SUP4*-CTC1 (CT₁CCAA), and CAA positioned upstream of the *SUP4*-CTC2 initiation region (*SUP4* alleles US1 and US2 in Table 1) did not produce elevated levels of in vivo suppression, indicating that adenosines at positions within the region -2 to -7 did not cause an increase in productive initiation events. Together, these data show that transcription start sites do not occur efficiently on *SUP4* templates at positions upstream of -1 or downstream of +3. The position of this 4-bp initiation window is likely determined by the precise placement of initiation factor TFIIB on the upstream DNA by the A-block binding component of TFIIC (3, 12, 20, 28, 37).

Purine- and pyrimidine-initiated transcripts compared. Our various substitution mutations (Table 1) replaced the wild-type AA sequence with either GC, YA, or TC. The first of these was without measurable effect, showing that RNA polymerase III can carry out all phases of initiation and early elongation with a GC-rich start site. The profound effects of the AA-to-TC mutations at the start site are attributable to the substitution of pyrimidine for purine in the transcript, rather than simply to the change in the percentage of A + T (see above). The following observations of in vitro transcription on *SUP4* templates with either AA or TC in the two crucial positions suggest to us a mechanistic basis for the requirement that transcription start with a purine nucleotide. (i) For optimal in vitro transcription, NTPs are required at higher concentrations if they are involved in the formation of the first phosphodiester bond. Transcription of *SUP4*-CTC2 in conditions of limiting CTP causes a reduced rate of in vitro product accumulation, while *SUP4* transcription kinetics are much less affected at the same limiting concentration of ATP. It is noteworthy that *SUP4* transcription has a very low apparent K_m for CTP, despite the fact that cytosine is required early (at position 3) in the growing *SUP4* RNA. This suggests that the initial dinucleotide made on *SUP4* (pppApA) acts to stabilize the newly formed elongation complex while that made on *SUP4*-CTC2 does not. (ii) Single-round transcription kinetic measurements showed that initiation and early elongation by polymerase III is faster on *SUP4* than on *SUP4*-CTC1 or *SUP4*-CTC2. This difference seems to be a direct consequence of the AA-to-TC change at the +1 and +2 nucleotide positions. (iii) Transcription of *SUP4*-CTC2 either in conditions of limiting ATP or in the absence of ATP generates an all-pyrimidine hexanucleotide RNA which appears to readily undergo transcript slippage and become repositioned by 3 nucleotides upstream. This shows that a 6-nucleotide-long all-pyrimidine RNA, pppCCUCUU, is not stably sequestered in the *SUP4*-CTC2 complex and raises the possibility that RNA polymerase binds more strongly to newly initiated transcripts that are purine rich. (iv) Binary preinitiation complexes and complexes in which the first phosphodiester bond had been preformed differed greatly in their abilities to generate heparin-resistant ternary complexes when challenged with a mixture of heparin and NTPs; *SUP4* complexes were able to generate high levels of heparin resistance, while those formed on *SUP4*-CTC2 were nearly completely inactivated. The ability of the *SUP4* complexes to successfully sequester and polymerize NTPs in the presence of a strong heparin challenge suggests that polymerase III transcription complexes containing nascent transcripts as short as 2 adenine nucleotides are functionally similar to highly processive postinitiation complexes. Cytidine-containing dinucleotides, on the other hand, seem less able to promote a transition from an unstable ini-

ation complex to a stable, elongation-competent state. (v) The oligonucleotides CpCpU and UpCpC, which correspond to start site sequences in *SUP4*-CTC2, are more readily released as abortive initiation products by RNA polymerase III than are the corresponding start site oligonucleotides from *SUP4*. This finding is also consistent with the notion that short, all-pyrimidine RNAs formed on *SUP4*-CTC2 do not remain bound to the polymerase III-template complex as avidly as do purine-containing transcripts generated on the *SUP4* gene.

The observations that transcription start site sequences show a strong bias for purines and that RNA polymerase from *E. coli* contains a purine-specific +1 binding site are generally reconciled by the presumption that having a purine residue present at the +1 position of the RNA supports more rapid formation of the first phosphodiester bond. However, our results suggest that after initiation but prior to stable elongation complex formation, the presence of a 5'-terminal purine nucleoside residue makes a further important contribution to transcription efficiency.

A mechanistic model for the deleterious effect of transcription initiation with pyrimidines. The observed properties of in vitro transcription from *SUP4*-CTC2 suggest that there may be an early kinetic competition between chain extension and the abortive process, so that a low rate of nucleotide addition during initiation favors increased release of short RNA products. Newly initiated transcripts of up to 6 nucleotides formed on *SUP4*-CTC2 are expected to exist as rPy:dPu heteroduplex. Heteroduplexes of this type are less stable than, and structurally distinct from, either the corresponding rPu:dPy heteroduplex or the rPu:rPy and dPu:dPy homoduplexes (7, 16, 27, 38, 40). However, there is some question as to whether these concepts of heteroduplex stability and structure arising from studies of long polynucleotides can be directly applied to the short nascent RNA in a newly formed transcription complex. We propose that purine ribonucleotide residues in the newly formed transcript confer additional complex stability due to more favorable interactions of these residues with a protein component of RNA polymerase. Following synthesis of the first phosphodiester bond, the 3' hydroxyl of residue 2 must be repositioned relative to the enzyme catalytic center to allow continued chain extension. This repositioning corresponds to a translocation of residue 2 from the $i + 1$ site to the i site (in the nomenclature of the *E. coli* system), with the concomitant placement of residue 1 in a new location, possibly the entry point of the RNA product binding site. The nature of these interactions makes the early transcription complex highly sensitive to the purine content of its initial RNA sequence; newly initiated transcripts with purine ribonucleotide 5' termini (or the short heteroduplex of those transcripts with DNA) are bound more stably at this product binding site than those containing 5' pyrimidine ribonucleotide residues. Given the demonstrated preference for purines over pyrimidines at the i site (47, 48) and the preference for purines over pyrimidines that we have proposed for the point of entry into the RNA product binding site, transcription complexes containing dinucleotide transcripts initiated at consecutive pyrimidines are expected to be less stable than either of the general types, pppPupPu or pppPupPy (pppPypPu was not observed). In agreement with this proposal, in vivo suppression levels from *SUP4*-CTC2 (pppPypPy initial dinucleotide) are much less than those from *SUP4*-CGC2 (pppPupPy initial dinucleotide), which are themselves slightly less than those from *SUP4* (pppPupPu initial dinucleotide). Our hypothesis regarding the importance of protein-nucleic acid interactions in stabilizing short transcripts in newly formed ternary complexes is supported by the finding that mutations in a region of the second-

largest subunit of yeast RNA polymerase III thought to be associated with RNA product binding (22, 25, 34) are able to compensate for the *SUP4*-CTC2 defect, completely restoring in vivo suppression to *SUP4* levels (50).

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