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# The γ subunit of DNA polymerase III holoenzyme of *Escherichia coli* is produced by ribosomal frameshifting

**(DNA replication/*dnaX*/*dnaZ*/translation/*lacZ* fusions)**

ANN M. FLOWER AND CHARLES S. MCHENRY*

Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center, Denver, CO 80262

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**ABSTRACT** **The τ and γ subunits of DNA polymerase III holoenzyme are both products of the *dnaX* gene. Since τ and γ are required as stoichiometric components of the replicative complex, a mechanism must exist for the cell to coordinate their synthesis and ensure that both subunits are present in an adequate quantity and ratio for assembly. We have proposed that γ is produced by a translational frameshift event. In this report, we describe the use of *dnaX–lacZ* fusions in all three reading frames to demonstrate that γ, the shorter product of *dnaX*, is generated by ribosomal frameshifting to the −1 reading frame of the mRNA within an oligo(A) sequence that is followed by a sequence predicted to form a stable secondary structure. Immediately after frameshifting a stop codon is encountered, leading to translational termination. Mutagenesis of the oligo(A) sequence abolishes frameshifting, and partial disruption of the predicted distal secondary structure severely impairs the efficiency. Comparison of the expression of *lacZ* fused to *dnaX* distal to the site of frameshifting in the −1 and 0 reading frames indicates that the efficiency of frameshifting is ≈40%.**

DNA polymerase III holoenzyme is the major replicative enzyme in *Escherichia coli*. It is a complex enzyme consisting of at least seven distinct subunits, each required for full reconstitution of holoenzyme activity *in vitro* (for a review, see ref. 1). The structural genes for five of the subunits have been identified and their nucleic acid sequences have been determined. The structural gene for the α subunit is *dnaE* (2, 3); *dnaN* is the gene for the β subunit (4, 5); ε is encoded by *dnaQ* (*mutD*) (6–10); and the τ and γ subunits are both encoded by *dnaX* (11–15).

Based on complementation, *dnaX* was originally defined as two distinct genes, *dnaX* and *dnaZ* (12). The *dnaZ* gene product was identified to be γ by *in vitro* complementation of inactive extracts of temperature-sensitive *dnaZ* mutants (11, 13) and by the overproduction of γ from an expression vector containing *dnaZ* (13, 16). The *dnaX* gene product was first identified to be τ based on the comigration of τ and the labeled *dnaX* gene product from "maxicells" during electrophoresis (14, 15). This conclusion was confirmed by immunoprecipitation of the *dnaX* gene product with a monoclonal antibody directed against τ (17).

A 2.2-kilobase restriction fragment can complement both *dnaZ* and *dnaX* mutations and direct the synthesis of both the γ and τ subunits (14, 15). In a single open reading frame, 2.2 kilobases can encode no more than 80,000 daltons of protein. The molecular weights of τ and γ are 71,000 and 52,000, respectively; the sum of these is clearly in excess of the coding capacity of the region. Deletion analysis and partial tryptic digests indicated that τ and γ share a common amino terminus (14, 15). Determination of the nucleotide sequence of the region demonstrated that the two proteins are encoded by a single continuous open reading frame (18, 19). Since *dnaZ* is contained within *dnaX*, it has been proposed that the use of *dnaZ* be discontinued and that the gene should be designated *dnaX* (20). A similar situation may exist in *Salmonella typhimurium*, where the *dnaZ* and *dnaX* genes (analogous to those in *E. coli*) are found to be genetically distinct by some criteria yet map within one another (21).

DNA polymerase III holoenzyme has been proposed to be an asymmetric dimer with functionally distinct leading- and lagging-strand polymerases (1, 17, 22–25). Both τ and γ are stoichiometric components of the same holoenzyme assemblies and appear to assemble into the polymerase by similar molecular interactions (17, 25). Analysis of the predicted protein sequences of τ and γ suggested that the carboxyl-terminal portion of τ exhibits similarity to many proteins that interact with nucleic acids (26, 27). We have proposed (26, 27) that τ might be associated with the highly processive leading-strand half of the polymerase, while γ, lacking the nucleic acid-binding domain, might be associated with the lagging-strand half. This proposal is supported by the observation that τ increases the processivity of DNA polymerase III (28).

The production of the related τ and γ subunits from a single open reading frame must be tightly regulated so that the proper quantity of each is synthesized. We have suggested that translational frameshifting leads to the generation of the shorter, γ product of *dnaX* (1, 26, 27, 29, 30). Here, we identify a site where ribosomes shift to the −1 ribosomal reading frame with 40% efficiency, leading to the production of the γ subunit.

## MATERIALS AND METHODS

**Strains and Plasmids.** All strains used were *E. coli* K-12 strains. Strains D1245 [*hsdS20*, *recA56*, Δ(*lac*)*74*, *rpsL20*, *proA2*, *ara-14*, *xyl-15*, *mtl-1*, *supE44*] and CJ236 [*dut*, *ung*, *thi*, *relA*, pCJ105(Cm$^R$)] were gifts of J. Betz and J. Brown of our department.

Plasmid pMWZ1101, containing wild-type *dnaX*, was constructed by M. Welch of this laboratory. Plasmid pRS552, used for protein fusions to β-galactosidase, was obtained from R. Simons (31). Plasmid pDM1, containing the bacteriophage f1 origin of replication, was obtained from B. Kemper (32). Plasmid pBBMD11 is an overproducer of *dnaX* products, inducible by isopropyl β-D-thiogalactopyranoside, that was constructed by M. Bradley of this laboratory.

Fusion plasmids pAF412, pAF440, pAF444, pAF452, and pAF483 were constructed by ligation of restriction fragments containing the appropriate *dnaX* sequences to pRS552 so that the reading frame of τ continued into *lacZ*, directing the expression of a τ–β-galactosidase fusion protein. A partial restriction map of *dnaX*, indicating the sites used for cloning, is shown in Fig. 1. pMWZ1101 was digested with the down-

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*To whom reprint requests should be addressed.

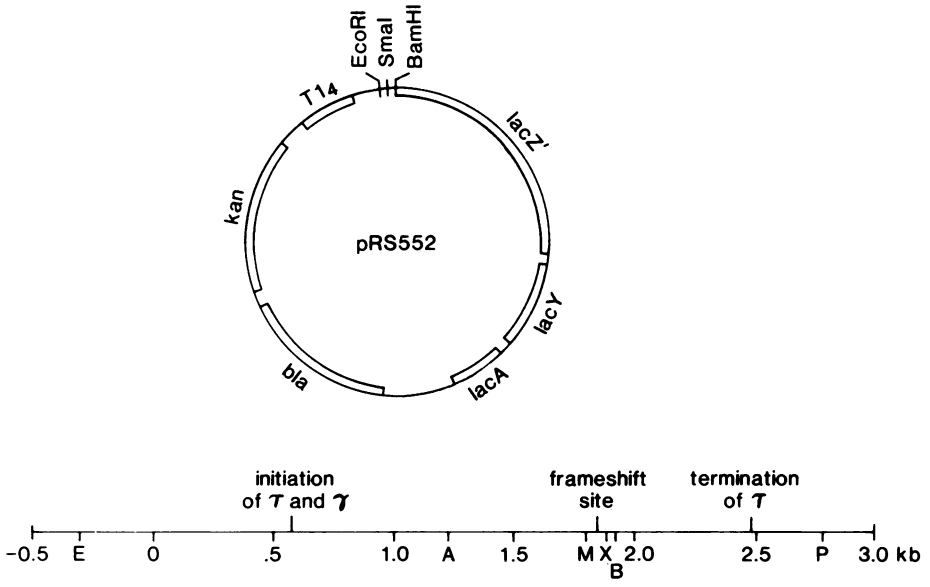


FIG. 1. Restriction maps of *E. coli dnaX* gene (*Lower*) and protein-fusion vector pRS552 (*Upper*). Numbering of the *dnaX* gene is as in ref. 18. $T1_4$ represents four tandem copies of the strong transcriptional terminator, T1, from the *rrnB* operon. Restriction sites: E, *Eco*RI; A, *Afl* II; M, *Pfl*MI; X, *Xma* III; B, *Bst*EII; P, *Pst* I. kb, Kilobases.

stream restriction enzyme [*Pfl*MI (pAF412), *Xma* III (pAF440), or *Bst*EII (pAF452)], then treated with either nuclease S1 (pAF412 and pAF440) or T4 DNA polymerase (pAF452) and digested with *Eco*RI. pRS552 was digested with *Bam*HI, treated with nuclease S1 (pAF412 and pAF440) or T4 DNA polymerase (pAF452), and digested with *Eco*RI. pAF444 and pAF483 were created by using the polymerase chain reaction (33) to amplify the desired portion of DNA and incorporate convenient restriction sites (for details of all of these constructions, see ref. 34). Fusions at codon 444 in the three reading frames were created by ligating the *Eco*RI–*Xma* III fragment from pMWZ1101, pRS552 cut with *Eco*RI and *Bam*HI, and synthetic oligonucleotides that contained *Xma* III and *Bam*HI ends. To create pAF4442 the sense oligonucleotide had the sequence 5'-GGCCGGTCGGAACAAC-CGG-3'. To construct pAF4441 the sequence of the sense oligonucleotide was 5'-GGCCGGTCGAACAACCGG (underlining denotes bases added to place *lacZ* in the proper reading frame).

**Immunoblots.** SDS/PAGE was carried out in 5–10% polyacrylamide gels in 0.1% SDS (35). Nitrocellulose "Western blot" detection of γ was performed as described by Renart *et al.* (36), using polyclonal antibody to γ (prepared by M. Bradley of this laboratory).

**Densitometric Quantitation of γ.** Nitrocellulose sheets were developed as described above and scanned on a Helena Quick-Scan R & D densitometer set up in the reflection mode (37). All γ determinations were performed within the linear range of the instrument. Purified γ was used as a standard.

**Enzyme Assays.** Exponentially growing cells were lysed by a modification of the procedure of Lupski *et al.* (38). β-Lactamase and β-galactosidase assays were performed as described (38, 39). Protein determinations were performed using the Bio-Rad Bradford assay system with γ-globulin as the standard.

**Site-Directed Mutagenesis.** Site-directed mutagenesis was performed by an adaptation of the method described by Kunkel (40). Phagemids were constructed by inserting a 534-base-pair *Sal* I restriction fragment from pDM1 containing the f1 origin of replication into the *Sal* I site of pAF4442, giving rise to pAF44421. An oligonucleotide containing the desired mutation was annealed and the primer–template was purified over a Bio-Gel A-5m (Bio-Rad) column equilibrated with 50 mM Hepes, pH 7.5/100 mM potassium glutamate/1 mM EDTA. The primer was extended by the addition of 0.5 mM ATP, single-stranded DNA-binding protein, all four dNTPs, and DNA polymerase III holoenzyme in a reaction buffer containing 50 mM Hepes (pH 7.5), 10 mM dithiothreitol, 100 mM potassium glutamate, and 10 mM $Mg(OAc)_2$. After elongation, T4 DNA ligase was added and the mixture was incubated at 37°C for 1 hr. The nucleotide sequences of plasmids from resultant transformants were determined to verify that the desired mutations had occurred.

## RESULTS

Sequence analysis of the *dnaX* gene demonstrated that τ and γ are encoded within the same continuous open reading frame (18, 19). There are several known mechanisms for production of two proteins from a single open reading frame: (*i*) γ could be produced by proteolysis of τ; (*ii*) mRNA processing could produce two distinct mRNAs, one encoding τ and the other γ; or (*iii*) ribosomal frameshifting could lead to the production of γ.

Our initial step toward elucidating the mechanism of γ production was to identify the minimal 3' *dnaX* sequence required for efficient γ biosynthesis. A series of *dnaX–lacZ* fusions was constructed that contained the amino-terminal portion of *dnaX* fused in the same reading frame to the carboxyl-terminal portion of *lacZ*. These plasmids expressed fusion proteins consisting of τ sequences fused to β-galactosidase and also expressed γ when all of the required sequence was present. When the 3' sequence required for γ production was deleted, only the fusion protein was synthesized, not foreshortened versions of γ. This provided advantages over deletion methods where a few necessary codons could be deleted without noticeably affecting the molecular weight of the protein produced. The production of γ was observed by SDS/PAGE followed by immunoblotting procedures using a polyclonal antibody directed against γ.

Cells containing the vector with no *dnaX* insert (pRS552) produced only the chromosomally encoded γ (Fig. 2 *Left*). Cells containing a fusion at codon 412 (pAF412) produced no more γ than pRS552, although this plasmid did express the predicted τ–β-galactosidase fusion protein. Plasmids with the fusion at codon 440 (pAF440) directed the synthesis of a small

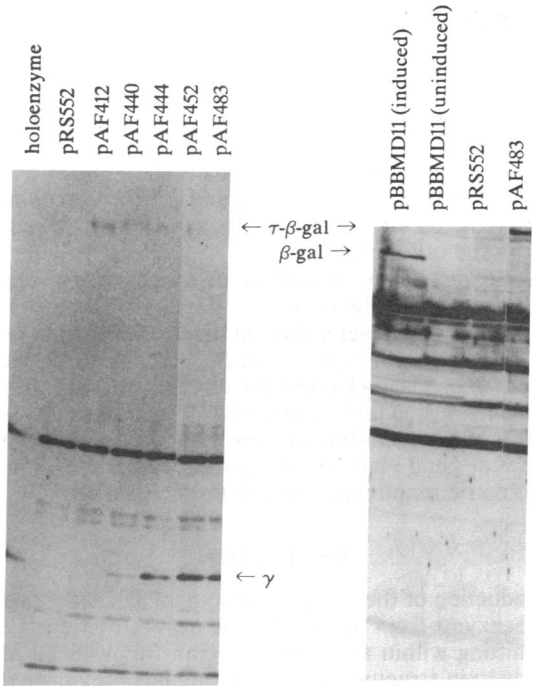


FIG. 2. (*Left*) Immunoblot of extracts from strain D1245 containing *dnaX–lacZ* fusions. Polyclonal antibody directed against γ was used. Left lane, holoenzyme marker. Refer to Table 1 for quantitation of γ band intensities. Although a large crossreacting species comigrating with τ is detected, we do not believe it is τ, since τ is present at only low levels in the cell (17). (*Right*) Immunoblot demonstrating that proteolysis of τ–β-galactosidase fusion protein (τ-β-gal) does not occur to produce γ. pBBMD11 (induced) produced natural β-galactosidase (β-gal). Polyclonal antibody directed against a RecA–reverse transcriptase–β-galactosidase fusion protein was used.

amount of γ, as well as fusion protein. Fusions at codons 444 (pAF444), 452 (pAF452), and 483 (pAF483) all produced γ at high levels. Therefore, the region between codon 412 and codon 440 contained the 3′-terminal sequence required for production of γ.

The possibility that proteolysis of τ produces γ was addressed by examining the products of these fusion plasmids (Fig. 2 *Right*). If a τ–β-galactosidase fusion protein is proteolyzed to produce γ, a shorter fusion protein containing the carboxyl-terminal portion of τ fused to β-galactosidase should also be produced. Using antibody directed against β-galactosidase, we were unable to detect such a proteolytically generated smaller fusion product in any of our experiments.

In related work directed toward mapping the *dnaX* operon, we found that no processing of mRNA occurs within *dnaX*. Endonuclease S1 digestion analysis was performed with probes that were radiolabeled either at the 5′ or the 3′ end and extended across the *dnaX* coding sequence (unpublished work). We did not detect any products that indicated processing of the mRNA.

Examination of the DNA sequence between codons 412 and 440 indicated the presence of only one out-of-frame stop

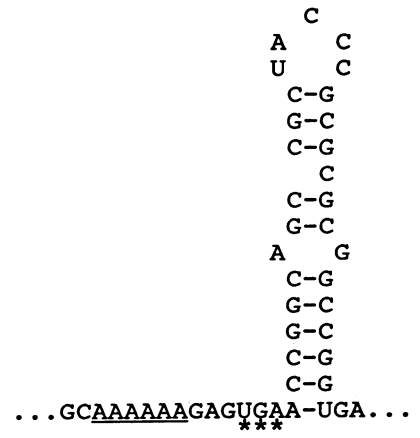


FIG. 4. Predicted stable secondary structure following oligo(A) region (underlined) in *dnaX* mRNA.

codon in this region (Fig. 3). This stop codon (in the −1 reading frame following amino acid 431) immediately follows a stretch of six adenines in the sequence A-AAA-AAG, followed by a region of potential secondary structure (Fig. 4). We identified this region as a site of potential ribosomal frameshifting based on analogy to retroviral frameshifting that occurs in homopolymeric regions followed by secondary structure (41–46). We predicted that during translation, the peptidyl-tRNA encoding AAA/lysine (codon 429) and the aminoacyl-tRNA reading AAG/lysine (codon 430) simultaneously slip in the 5′ direction by one nucleotide, reading AAA/lysine and AAA/lysine, respectively. The same $tRNA^{Lys}$ (anticodon UUU) is used to read both AAA and AAG. This slippage would allow translation to continue in the −1 reading frame until a stop codon was encountered after the incorporation of one more amino acid.

To test our hypothesis, we used fusion plasmid pAF444, which expressed a high level of γ, and altered the reading frame of *lacZ* to −1 or +1 relative to *dnaX* by oligonucleotide replacement cloning techniques, giving rise to pAF4441 (+1 frame) and pAF4442 (−1 frame). As expected, due to the presence of an intervening stop codon, neither of these plasmids expressed β-galactosidase (data not shown). We used site-directed mutagenesis to alter the predicted γ stop codon to a sense codon (TGA to CGA), producing plasmid pAF4443. The amino acid sequence of τ was not altered by this change. A restriction fragment containing this mutation was also cloned into the 0-frame and +1-frame vectors (pAF444 and pAF4441), giving rise to plasmids pAF4445 and pAF4446, respectively. If frameshifting occurred, this mutation would allow translation in the −1 reading frame to continue past the site of the mutated stop codon into *lacZ* in the proper reading frame for β-galactosidase expression. The increase in β-galactosidase activity (Table 1) and the appearance of the large fusion protein in the immunoblots from the mutants (Fig. 5) supported our hypothesis. Furthermore, γ was no longer made from these fusions because the normal −1-frame stop codon used for production of γ had been altered. Plasmids pAF4445 and pAF4446 produced short fusion proteins of the predicted molecular weights (49,000

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                GUGCUGGCGGCGCGCCAGCAGUUGCAGCGCGUGCAGGGAGCAACCAAAGCAAAAAAGAGUGAACCGGCAGCCGCUACCCGCGCGCGG
                                                                    ***
 codon  412                  420                  430                  439
 0      ValLeuAlaAlaArgGlnGlnLeuGlnArgValGlnGlyAlaThrLysAlaLysLysSerGluProAlaAlaAlaThrArgAlaArg
+1       CysTrpArgArgAlaSerSerCysSerAlaCysArgGluGlnProLysGlnLysArgValAsnArgGlnProLeuProAlaArg
-1        AlaGlyGlyAlaProAlaValAlaAlaArgAlaGlySerAsnGlnSerLysLysGluTRM
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FIG. 3. Nucleotide and predicted amino acid sequences between codons 412 and 440 of *dnaX*. Underlined nucleotides mark the oligo(A) region and asterisks mark the −1 stop codon. TRM indicates the −1 stop codon.

Table 1. Expression of $\gamma$ and $\beta$-galactosidase from *dnaX–lacZ* fusion plasmids

| Plasmid | Specific activity* | | $\beta$-Galactosidase/ $\beta$-lactamase | $\gamma$, ng† |
|---|---|---|---|---|
| | $\beta$-Galactosidase | $\beta$-Lactamase | | |
| pRS552 | <10 | 907 | <0.01 | 0 |
| pAF412 | 344 | 911 | 0.38 | 0 |
| pAF440 | 273 | 682 | 0.40 | 1.2 |
| pAF444 | 139 | 884 | 0.16 | 3.0 |
| pAF452 | 149 | 968 | 0.15 | 3.3 |
| pAF483 | 105 | 1118 | 0.09 | 4.8 |
| pAF4443 | 123 | 1042 | 0.12 | 0 |
| pAF4447 | <10 | ND | — | — |
| pAF4448 | <10 | ND | — | — |

ND, not determined.
*Units per mg of total cell protein.
†Per $10^3$ $\beta$-lactamase units, determined by densitometry of immunoblots.

and 54,000) due to continued frameshifting and translation into a nonproductive reading frame of *lacZ* (Fig. 5).

To test our prediction that frameshifting occurs at the oligo(A) at codons 429 and 430, we altered the adenine residue at the 3′ position of codon 429 to a thymine or a cytosine. If frameshifting is able to occur due to slippage of two lysine tRNAs, these mutations should prevent slippage and thereby prevent frameshifting. Synthetic restriction fragments containing the mutations were cloned into pAF4443, giving rise to pAF4447 (A → T) and pAF4448 (A → C). Both mutations reduced the amount of $\beta$-galactosidase expressed to undetectable levels (Table 1), confirming our prediction.

Quantitation of $\gamma$ and $\beta$-galactosidase produced by the various fusions demonstrated that the efficiency of frameshifting increased as the fusion contained more 3′ *dnaX* sequence beyond the site of frameshifting (Table 1). Assuming a constant rate of translational initiation at the amino terminus of *dnaX* mRNA, we expected a decrease in $\beta$-galactosidase expression concomitant with an increase of $\gamma$ production. As shown, pAF444 is 2.5-fold more efficient at frameshifting than pAF440; pAF452 is 2.8-fold more efficient; and pAF483 is 4-fold more efficient. This observed increase in efficiency of frameshifting can be correlated with predicted secondary structures downstream from the frameshifting location. The fusion at codon 440 (pAF440) interrupted five base pairs of a very stable stem-and-loop structure following the site of frameshifting (Fig. 4). In pAF444 and pAF452, this structure remained intact and $\gamma$ was produced much more efficiently. Plasmid pAF483 contained a second predicted stem-and-loop structure (1) that was not present in the other plasmids.

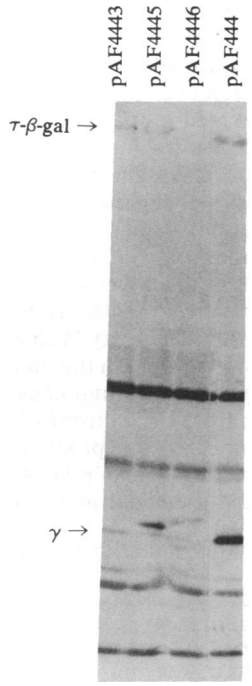


FIG. 5. Immunoblot of extracts from cells containing *dnaX–lacZ* fusions with a mutation of the −1 stop codon.

An indication of the efficiency of frameshifting was obtained by comparing the $\beta$-galactosidase activity of pAF444, which produced $\beta$-galactosidase in the 0 reading frame, with that of pAF4443, which produced $\beta$-galactosidase in the −1 reading frame. Plasmid pAF4443 directed the synthesis of 70% as much fusion protein as pAF444, indicating an overall efficiency of frameshifting of 40%. Thus, nearly equimolar quantities of $\gamma$ and $\tau$ will be synthesized, consistent with their stoichiometric requirement within the holoenzyme.

## DISCUSSION

The production of the $\tau$ and $\gamma$ subunits of DNA polymerase III holoenzyme from the *dnaX* gene occurs by translational frameshifting within an oligo(A) region followed by an essential hairpin structure. Frameshifting has previously been shown to be an important mechanism for the regulation of protein production in *E. coli*. The peptide-chain-release factor 2 (RF2) autoregulates its expression through a frameshift mechanism (47, 48). The structural gene encoding RF2 contains an in-frame UGA stop codon recognized exclusively by RF2. When RF2 is present in excess, translation is terminated. If RF2 levels are low, frameshifting occurs, resulting in the production of the functional RF2 product. Another example of frameshifting in *E. coli* has been demonstrated for the production of the transposase encoded by insertion sequence IS*1*. This frameshift results in the production of a InsA–B′–InsB fusion protein, an event that probably does not occur with high frequency (49).

The frameshift that occurs in *dnaX* is remarkably similar to that found in several retroviruses (41–46) and in IS*1* (49). These all contain a homopolymeric stretch followed by a region of secondary structure. It has been proposed that when ribosomes encounter the homopolymeric region, the peptidyl-tRNA and the aminoacyl-tRNA simultaneously slip in the 5′ direction by one nucleotide (−1 frameshift) (43). The relative importance of each base in the frameshift region for Rous sarcoma virus has been examined by site-directed mutagenesis (43). The 3′ nucleotide of the codon recognized by the shifting peptidyl-tRNA and the three nucleotides in the codon recognized by the incoming aminoacyl-tRNA are the most critical for determining frameshift efficiency. Our results indicate that the mechanism of frameshifting in *dnaX* is analogous to that in retroviruses, as we altered the 3′ nucleotide of the peptidyl-tRNA codon and observed a dramatic decrease in the efficiency of frameshifting.

Retroviral frameshifting and the IS*1* frameshift event have been shown to be dependent on mRNA secondary structure distal to the actual site of frameshifting (41–46, 49, 50). Our results are consistent with these models, as disruption of predicted stem-and-loop structures severely impaired the efficiency of the *dnaX* frameshift. The oligo(A) region is followed by a predicted stem-and-loop structure with a calculated free energy of −28.4 kcal (51) (Fig. 4), and a second stem-and-loop structure (1) that also seems to affect the ability of the ribosomes to frameshift is located further downstream.

The protein product of the *dnaX* frameshift, $\gamma$, is a 431-amino acid protein with a deduced molecular weight of 47,544. The first 430 of these amino acids will be identical to

the amino-terminal 430 amino acids of $\tau$. This mechanism of gene expression provides the cell with a method for producing two related essential products in nearly equivalent quantities. This is an example of ribosomal frameshifting that (*i*) leads to translational termination, rather than to translation of a continuous open reading frame, and (*ii*) allows the expression of two distinct proteins from the same open reading frame at nearly equivalent levels.

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