central inhibition and the anteriorly directed AN reflex will act synergistically, preventing the SR_1-SEMN #2 segmental resistance reflex.

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Externally Suppressible Proline Quadruplet CCC$^{U}_{\bullet}$

Abstract. *Three (+1) frameshift mutations located at different genetic sites respond with high specificity to the same external suppressor. In each case, the suppressor restores small amounts of protein that is normal in electrophoretic mobility and heat stability. One of these proteins has been shown to have the wild-type amino acid sequence. The messenger RNA quadruplet CCC$^{U}_{\bullet}$ appears to be common to all three frameshift sites and to be translated by the suppressor as proline. A likely suppressor agent is a proline transfer RNA with a quadruplet anticodon or its functional equivalent.*

We have reported external suppression of a frameshift mutation, *hisD3018*, in the histidinol dehydrogenase gene of *Salmonella* (*1*). This frameshift was induced with the frameshift mutagen ICR-191 (*2*) by Oeschger and Hartman (*3*). It is revertible not only by ICR compounds but also by certain alkylating agents such as DES (*2*) and NG (*2–4*). The *3018* frameshift is a (+1) type (*2*), most likely containing an extra C in a repeating messenger RNA (mRNA) (*2*) sequence of C residues (*2, 4, 5*). External suppressors of *3018* are efficiently induced by ICR-191 (*1*). Suppression restores small amounts of histidinol dehydrogenase with the wild-type amino acid sequence; this suggests that the suppressors read the (+1) mRNA sequence inefficiently as the correct sequence (*6*). Riddle and Roth showed that most frameshifts which are revertible by alkylating agents as well as ICR compounds, tentatively classified as (+1) types, are externally suppressible. Two classes of externally suppressible frameshift, almost all induced with ICR-191, were delineated. Suppressors of one class of frameshift do not suppress the other (*7*). These frameshifts may be (+1) additions in different types of repeated sequences in mRNA. We have tried to pinpoint the suppressible mRNA sequence and to characterize the proteins that result from suppression of other frameshifts of the *3018* class located at different sites in the histidinol dehydrogenase gene. One such frameshift, *3749*, was found to have an extensive (+1) mRNA sequence of six or seven residues in common with *3018* and to produce histidinol dehydrogenase which is normal in electrophoretic mobility and heat stability (*8*). We have now investigated a third frameshift of this type, *2565*. The accumulated data implicate a suppressor that enables the quadruplet CCC$^{U}_{\bullet}$ (*2*) to be translated as proline.

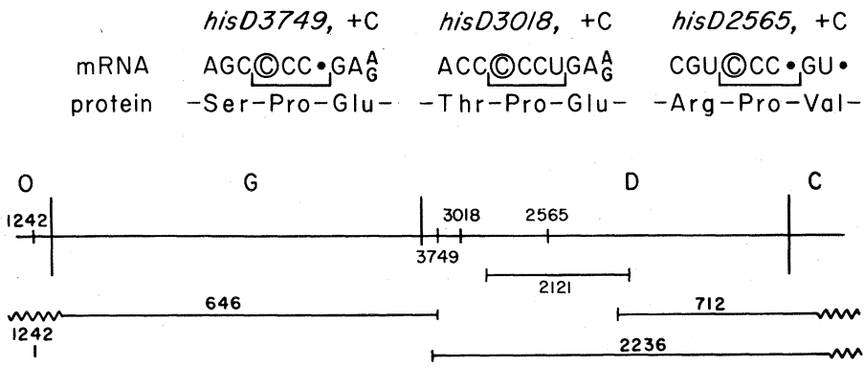

Fig. 1. Genetic sites and nature of cross-suppressible frameshifts of the *3018* class in the *hisD* gene. The *3749* site maps under deletion *646* (*7*). The *3018* site maps between deletions *646* and *2121* (*3*). The *2565* site is yet more internal in the gene and maps under *2121*. The inferred similar sites of suppression in *3018*, *3749*, and *2565* mRNA are shown above the map. An extra C (circled) in a repeated sequence of C residues is considered to be the basis of each (+1) frameshift. To restore normal protein the suppressor reads the quadruplet CCC$^{U}_{\bullet}$ as proline.

Frameshift mutations were induced with ICR-191 in strain *his01242* (*2*) and selected for strong polarity (reduced expression of genes following the mutant gene in the histidine gene cluster) by the temperature method (*9*). Independent cultures of *his01242* in 2 ml of histidine-supplemented 2EM medium (*2*) containing 20 μg of ICR-191 were grown overnight on a shaking device at 37°C; a portion (0.1 ml) of each culture was plated on histidine-supplemented E agar containing 2 percent glucose. ICR-191 (10 μg) was spotted on each plate to enhance mutagenesis further, and the plates were incubated for 72 hours at 43°C. Polar *his* mutants, recognized by their smooth colony morphology, were isolated and examined. Of 24 independent frameshift mutations isolated in the histidinol dehydrogenase gene, 13 showed the reversion and suppression properties of the *3018* class. These map in three different segments of the *hisD* gene, as determined by deletion mapping: three under *his-646*, as *3749*; five between *his-646* and *his-D2121*, as *3018*; and five under *2121*, including *2565* (Fig. 1). It is likely that all are similar (+1) mutations at three hotspots (sites where such mutations easily occur), and that each hotspot contains a repeated sequence of DNA GC pairs (*2*). Frameshifts at the *2565* site represent a previously uncharacterized set of mutations, and *2565* was selected for further study.

The altered mRNA sequence in *2565* was reconstructed with the in vitro codon assignments (*10*) by analysis of

amino acid changes in prototrophic double mutant histidinol dehydrogenase. It was necessary to examine at least two different double mutants to establish which mutation is shared in common and is therefore parental. The enzymes from revertants R1 and R47 were different in specific activity from wild type and from one another and they were also amenable to purification. These double mutant enzymes were therefore purified in quantity by the standard procedure (*11*) and compared to the wild type for changes by mapping of tryptic and chymotryptic peptides. Tryptic peptide T14 and the associated chymotryptic peptide C1 were altered in both revertants. For sequence analysis these peptides were purified in quantity, and the critical sequences were determined directly or by analysis of subtilisin fragments from each (see Table 1).

The wild-type sequence -Val-*Thr-Arg*-Pro-Val- (*2*) was altered to -Val-*Thr-Leu*-Pro-Val- (*2*) in R1 and -Val-*Arg-Val*-Pro-Val- (*2*) in R47 (the affected region is italicized) (Table 1). The observed amino acid changes establish the parental *2565* frameshift, like *3018* and *3749*, as a (+1) type. Because of code degeneracy and the small sample of double mutants examined, we cannot unequivocally define the (+1) addition. On formal grounds, however, an addition of a C residue where two to three mRNA C residues are repeated is most likely. The compensating frameshifts of R1 and R47 both appear to be (−1) deletions (*2*). A deletion of a G residue (*2*) under strong selective pressure may be the basis for the R1 frameshift, and a deletion of an A or the preceding ● residue (*2*) for the R47 frameshift. The outstanding feature common to *3018*, *3749*, and *2565* mRNA is the reconstructed sequence CCC$^{U}_{\bullet}$G at the frameshift site (Fig. 1).

When *3018* or *3749* carry the external suppressor, suf2, weak prototrophy results from the restoration of low levels of histidinol dehydrogenase activity (*6*, *7*) (Table 2). The restored enzyme is normal in electrophoretic mobility and in heat stability (*6*, *8*). It is one of these, the *3018suf2* (TR936) enzyme, that has been shown to have the wild-type amino acid sequence (*6*). A *2565suf2* strain was constructed by transduction of the suppressor gene into the mutant as recipient. Again, the suppressor restored small amounts of electrophoretically normal, heat-stable enzyme to the similar *2565* frameshift (Table 2 and Fig. 2).

It is very likely that the *3018* suppressors act during translation rather than transcription. The fact that ICR-191, a frameshift mutagen, induces *3018* suppressors (*1*, *6*) favors an altered (+ or −) (*2*) nucleic acid component of the translation system as the suppressor agent. A protein altered by frameshift mutation, such as RNA polymerase, would generally be expected to be produced as an inactive fragment. We have hypothesized that

Table 1. Reconstruction of altered mRNA sequence in frameshift *2565* from amino acid changes in (− +) double mutant histidinol dehydrogenase. Messenger triplets are shown above the specified amino acid residues in the protein. The *2565* frameshift is considered to insert an extra C residue in mRNA, the compensating frameshifts of R1 and R47 to delete single nucleotide residues as shown in brackets. Unless otherwise noted, peptides were purified by column chromatography. Peptide C1′ was produced from T14 as described (*4*). Subtilisin fragments were produced from T14 or C1′ and purified as described (*8*). Peptide T14NH$_2$ of R47 corresponds to the NH$_2$-terminal section of wild-type T14, and T14COOH of R47 corresponds to the COOH-terminal section. Only pertinent sections of these peptides are shown. Relative amounts of residues from each peptide before and after the Edman degradation (the step is shown in parentheses) or after digestion with carboxypeptidase B (CPB) are given. The average amount of peptide (in micromoles) for each analysis was: wild type: T14S1, 0.175; T14S2, 0.027; T14S3, 0.034; R1: T14C1′S1, 0.046; T14S2, 0.012; R47: T14NH$_2$, 0.075; T14-COOH, 0.040; C1, 0.023.

			Wild-type strain				
mRNA		GU•	ACG	CGU	CC•	GU•	
Protein		Val	Thr	Arg	Pro	Val	Ser
T14S1				Arg	Pro	Val	
				1.06	1.07	1.00	
Edman				(1)0.22	(2)0.12		
T14S2			Thr	Arg	(Pro	Val)* †	
			0.53	1.01	1.09	1.00	
Edman			(1)0.22	(2)0.60			
T14S3		Val	Thr	(Arg	Pro	Val)*	
		1.48	0.90	1.00	1.06		
Edman		(1)0.98	(2)0.48				
			R1 strain				
				−G	+C		
mRNA		GU•	ACG	C U C	CC•	GU•	
Protein		Val	Thr	Leu	Pro	Val	Ser
T14C1′S1			Thr	Leu	Pro	Val	Ser‡
			0.63	0.94	0.90	1.00	0.92
Edman			(1)0.32	(2)0.52	(3)0.45	(4)0.60	
T14S2		Val	(Thr	Leu	Pro	Val	Ser)§
		1.20	0.71	1.00	1.15		0.85
				R47 strain			
			−A		+C		
mRNA		GU•	CGC	GU C	CC•	GU•	
Protein		Val	Arg	Val	Pro	Val	Ser
T14NH$_2$	Gln	Val	Arg				
	2.10	1.00	1.00				
CPB	0.27	0.61	1.00				
T14COOH				Val	Pro	Val	Ser
				3.88	4.37		3.61
Edman				(1)3.04	(2)3.30	(3)2.10	(4)2.85
C1		Val	(Arg	Val)	(Pro	Val)*	
		2.70	1.19		1.00		
Edman		(1)1.96	(3)0.67	(3)1.32			

* Extracted from peptide maps stained with 0.025 percent ninhydrin (*8*). † Stains yellow. ‡ Isolated by paper chromatography and electrophoresis. § Extracted from peptide maps stained with 1 percent ninhydrin. Stains blue with substantial loss of NH$_2$-terminal valine.

Table 2. Enzyme levels in suppressed and unsuppressed strains. All strains carry the operator mutation *his01242* (*2*). Cells were grown to mid-log phase on E medium containing 15 μg of L-histidine per milliliter. Enzymes in crude extracts were assayed as previously described (*13*). The spectrophotometric method was used for analysis of histidinol dehydrogenase. Specific activity was calculated as the number of units of enzyme per milligram of protein and the activity of the wild type was assigned a value of 100 percent in each experiment. Values given in equivalent order were obtained in the same experiment.

	Specific activity			
	Wild type (%)		Increase (%)	
	hisD	*hisC*	*hisD*	*hisC*
his01242	100, 100	100, 100		
Wild-type control				
hisD3749	0.2	3.1		
*hisD3749*suf2	1.3	9.3	1.1	6.2
hisD3018	0.3, 0.1	2.5, 4.3		
*hisD3018*suf2	2.0, 1.0	8.2, 8.5	1.7, 0.9	5.7, 4.2
hisD2565	0.1, 0.1	7.9, 5.6		
*hisD2565*suf2	1.6, 0.9	6.5, 5.4	1.5, 0.8	−1.4, −0.2

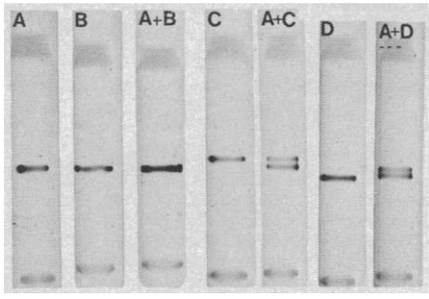

Fig. 2. Electrophoretic mobility of *2565*-suf2 histidinol dehydrogenase. Standard, *p*H 9.5, polyacrylamide gels were stained for histidinol dehydrogenase. For this experiment enzyme from *2565*suf2 cells, grown in 3 liters of E medium, was purified through the DEAE-Sephadex A-50 step by the standard procedure (*11*). Appropriate genetic tests showed the cells to be bona fide *2565*suf2 genotypes, containing no detectable intragenic revertants. All other samples used were crystalline preparations. The samples are: A, wild type, 6 μg; B, *2565*suf2, 300 μg; C, *497*R56, 10 μg. This enzyme lacks a glumatic acid residue (Ino, Hartman, and Yourno, unpublished results) and is, therefore, slower than wild type at *p*H 9.5; D, *3018*R95, 6 μg. This enzyme lacks an arginine residue (*14*) and is, therefore, faster than wild type at *p*H 9.5. Normal mobility appears in A, B, A + B, A + C, and A + D; C is above this line and D is below.

the suppressor is a tRNA with a quadruplet (+1) anticodon or a (+ or −) tRNA which is susceptible to slipping along a repeated mRNA sequence (*1*, *6*). This question can best be answered by direct isolation and characterization of the suppressor agent.

The accumulated data strongly suggest that the suppressor reads the quadruplet $CCC^{U}_{\bullet}$ as proline in each case (Fig. 1). If the overlapping quadruplets $^{U}_{\bullet}GA^{A}_{G}$ or •GU• were read instead by the suppressor as glutamic acid, the suppressed *2565* enzyme would carry a Val → Glu substitution and have greater than normal electrophoretic mobility at *p*H 9.5. The *3018* and *3749* mRNA's contain a $^{U}_{\bullet}GA$ codon in the (+) phase, overlapping the (+1) frameshift site. Possibly a nearby chain-terminating codon (UGA) plays some critical rephasing or rate-limiting role in suppression. However, the closest potential chain-terminating codon in *2565* is •(AG) (UAG) one codon removed from the frameshift site. In each case the quadruplet $CCC^{U}_{\bullet}$ at the frameshift site on mRNA is followed by a G residue. Again it is not clear whether this plays some role such as phasing in suppression. Whatever the detailed mechanism, the data indicate that the net effect of suppression is translation of the quadruplet $CCC^{U}_{\bullet}$ as proline.

About 1 percent of normal amounts of histidinol dehydrogenase are restored in each case by suppression. Yet about 5 percent more aminotransferase, product of the following gene, *hisC*, is produced in suppressed *3018* and *3749* strains (*7*). Suppressed *2565* strains do not produce measurably greater amounts of aminotransferase, which may therefore also be increased by 1 percent (Table 2). We do not understand the basis of this apparent hyperrelease from polarity in suppressed *3749* and *3018*. On the assumption that the enzyme assays are reliable, the hyperrelease may be due to reinitiation of mRNA reading after the frame shift site, dependent on the chain-terminating UGA codon generated at that point.

In summary, our results suggest that the quadruplet $CCC^{U}_{\bullet}$ is a sufficient if not necessary condition for external suppression of the *3018* class of frameshift. This is an exception to the triplet genetic code (*10*). However, the data do not rigorously define the specificity limits of the *3018* suppressors. A requirement for neighboring sequences, particularly chain-terminating codons, cannot be completely ruled out. Nor is it certain that the suppressors are absolutely specific for (+1) frameshifts in mRNA sequences of repeated C residues. The study of frameshift suppression is of considerable importance to further understanding of the genetic code and its translation into polypeptide structure.

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2. The following abbreviations are used: tRNA, transfer RNA; mRNA, messenger RNA; ICR-191, 2-chloro-6-methoxy-9-[3-(2-chloroethyl)-aminopropylamino]acridine dihydrochloride; DES, diethyl sulfate; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; C, cytidylate; G, guanidylate; U, uridylate; A, adenylate; $^{A}_{G}$, either A or G; $^{U}_{C}$, either U or C; •, unspecified nucleotide residue; $^{U}_{\bullet}$, either U or •; (+1) frameshift, the addition of a single nucleotide pair in DNA and resultant addition of a single nucleotide residue in mRNA; (−1) frameshift, the deletion of a single DNA nucleotide pair and a single mRNA nucleotide residue; GC pair, a deoxyguanidylate-deoxycytidylate nucleotide pair in DNA; E medium, minimal salts medium of Vogel and Bonner [as described in (*1*)]; 2EM, E medium containing 2 percent by volume of Difco liquid nutrient broth; *his*n, a mutation involving histidine biosynthesis; *hisD*, the histidine *D* (histidinol dehydrogenase) gene; *hisO*, the histidine operator gene; Val, valine; Thr, threonine; Arg, arginine; Pro, proline; Glu, glutamic acid; Leu, leucine; Gln, glutamine; and Ser, serine.
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Membrane Continuity between Plasmalemma and Nuclear Envelope in Spermatogenic Cells of Blasia

Abstract. *Ultrastructural study of liverwort antheridia showed that the spherical nuclei of some late-stage androgonial cells lie close to or appressed to the cell walls. In some cells the outer membrane of the nuclear envelope curves toward the wall and continues without interruption around the cell periphery as the plasmalemma. Subject to its confirmation as a natural occurrence, this evidence appears to support Robertson's concept of cellular organization.*

A modern, well-known concept of general cellular organization in part postulates continuity of the cytoplasmic membrane system (Robertson, *1*). According to this view, the continuous nature of nuclear envelope, endoplasmic reticulum, and plasmalemma membranes forms one or more open channels between the cell exterior and the so-called perinuclear space within the nuclear envelope. Such channels, even if intermittent in appearance, would play