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Influence of the *relA* gene on ribosome frameshifting

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Abstract We have examined the influence of genotype at the *relA* locus on the kinetics of leftward (or –1) frameshifting at a variety of codons calling for a limiting aminoacyl-tRNA species. We used *lacZ* left-frameshift reporter constructs carrying the sequence U UUC xyz, where xyz was each of three triplets coding for three different amino acids; we slowed the ribosomes at each of these by limiting for the amino acid or for the aminoacyl-tRNA. In all cases, limitation stimulated leftward frameshifting. In all cases, the stimulation was greater in *relA* mutant cells than in their wild-type *relA*⁺ counterparts. In the latter genotype, the increased frameshifting was constant from the start of the limitation regime. This was also true of the *relA* mutant strain during limitation for lysine-tRNA or for leucine; however, during limitation for isoleucine-tRNA (or for isoleucine) the mutant showed a gradual, progressive increase in frameshifting, suggesting an indirect effect. We suggest that gradual accumulation of undermodified tRNAs, which is characteristic of the *relA* response, is involved. However, the specific modification involved is unknown. It is not queosine: analysis of a *tgt* mutant that is completely defective in queosine modification showed no increase in leftward frameshifting on the reporter which showed the larger, gradual increase during the *relA* response to isoleucine-tRNA limitation.

Keywords Ribosomal frameshifting · Stringent response · Amino acid limitation · *relA*

Introduction

Long ago, we observed that aminoacyl-tRNA limitation could in some circumstances elicit phenotypic suppression of frameshift mutant alleles (Gallant and Foley 1980; Weiss and Gallant 1983, 1986; Kurland and Gallant 1986). The effect was particularly marked in *relA* mutant cells, which also show enhanced missense error frequencies at “hungry” codons calling for a limiting aminoacyl-tRNA. We took this finding as consistent with a mechanism of noncognate aminoacyl-tRNA binding at “hungry” codons, followed by ribosome frameshifting (Kurland 1979; Kurland and Gallant 1986).

In subsequent studies, we used a *lacZ* reporter system to define the molecular basis of phenotypic frameshifting (reviewed by Farabaugh 1996, 1997; Gallant and Lindsley 1993; Gallant et al. 2000). In the case of leftward (or –1) frameshifting, at least, these studies led to a simple molecular model: peptidyl-tRNA on the 5′ side of the “hungry” codon slips leftward (or 5′-ward) by one base, where the message sequence permits it to do so while retaining complementary pairing; this slippage permits the triplet overlapping the hungry codon from the left or 5′ side to be read by its cognate aminoacyl-tRNA, thus initiating the ribosome frameshift (Kolor et al. 1993; Barak et al. 1996).

This mechanism differs from the one we originally favored, and does not involve the binding of a noncognate ternary complex at any stage. Hence, the meaning of the *relA* effect must be different from what we originally supposed. We have accordingly reinvestigated the role of *relA* in ribosome frameshifting, using the *lacZ* reporter system. The reporter system uses a modified *lacZ* gene on a plasmid, in which synthesis of active β-galactosidase requires a leftward frameshift within the first 11 codons of the coding sequence. This region of the

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mRNA contains a site at which we have previously demonstrated high-frequency starvation-promoted frameshifting: the slippery sequence U UUC, on which peptidyl-phe-tRNA can slip leftward while retaining complementary pairing, followed by a triplet at which we manipulate the availability of aminoacyl-tRNA (Barak et al. 1996). In the experiments described here, we have assessed the kinetics of frameshifting at a variety of hungry codons in this sequence, in isogenic strains differing only at the *relA* locus.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains utilized in all experiments were derivatives of the *E. coli* strains CP78 (*thr leu his arg thi*) and CP79 (*thr leu his arg thi relA2*) (Fiil et al. 1968) with a complete deletion of the *lac* operon *lac* MC 1000 (Silhavy et al. 1984). Isogenic strains SJ 1502 (*tgt* wildtype *relA*) and SJ 1505 (*tgt relA*) were generously provided by Dr. Helga Kersten. Bacteria were cultivated in M63 medium (Silhavy et al. 1984) supplemented with 2 g/l D(+) glucose, 200 mg/l L-threonine, 0.1 mg/l thiamine, 100 mg/l each of L-histidine, L-arginine, and L-leucine. Selection for plasmid maintenance was imposed by addition of carbenicillin at a concentration of 1 mg/ml. All methods of extract preparation and enzyme and protein measurements were performed as previously described (Peter et al. 1992; Lindsley and Gallant 1993; Barak et al. 1996).

Exponentially growing cells were induced at $OD_{690}=0.2$, corresponding to approximately 40 µg/ml protein, by the addition of 2.5 mM cAMP and 2 mM isopropyl-β-D-galactopyranoside (IPTG), as previously described (Barak et al. 1996). Simultaneously, an aliquot was subjected to limitation for individual aminoacyl-tRNAs (aa-tRNA) by inhibiting the aminoacyl-tRNA synthetase with the corresponding hydroxamates; or limited for a required amino acid by replacing it by a growth-limiting concentration of the corresponding amino acid methyl ester (see Table 1).

Plasmid construction

Constructs were made by annealing the synthetic nucleotides 5'-AGC TTT TTC XYZ GTA TAG G-3' and 3'-AA AAG XYZ

CAT ATC CCT AG-5' and ligating the double-stranded oligonucleotide into the polylinker region of pMLB1115. Here XYZ represents the "hungry codon". XYZ was varied to allow the creation of frameshift sites with various different triplets following the slippery quadruplet (Table 1). Complementary pairs of synthetic oligonucleotides with sticky *Hind*III and *Bam*HI overhangs were ligated into pMLB1115 (see Barak et al. 1996) cut with those restriction enzymes. Plasmids containing this reporter gene were transformed into the *lac*-deletion host strains CP78 (*relA*⁺) and CP79 (*relA*). Methods of plasmid construction, transformation, and restriction analysis were as previously described (Lindsley and Gallant 1993). All constructs were verified by DNA sequencing.

Enzyme and protein assay

Samples (2–8 ml) of the growing culture were withdrawn, cooled on ice, and stored overnight at 0°C. The cells were then pelleted, washed once, resuspended in a volume of 0.4 ml, and sonicated at a protein concentration of 1–2 mg/ml, after which cell debris was centrifuged out and the supernatants were stored for assay. For β-galactosidase activity, up to 50-µl aliquots were mixed with *o*-nitrophenyl-galactoside (0.8 mg/ml) in 0.05 M sodium phosphate buffer (pH 7.0) in a final volume of 300 µl in a microcuvette (1 cm light path), and the change in absorbance at 420 nm was recorded. One enzyme unit is defined as the quantity yielding a change of 0.001 OD units per min. Protein was measured by the Folin method (Lowry et al. 1951).

Results

The frameshift reporter system

In all the constructs synthesis of active β-galactosidase depends upon leftward frameshifting. Each construct contains the shifty quadruplet U UUC (Kolor et al. 1993; Barak et al. 1996) followed by a triplet encoding an aminoacyl-tRNA which we make limiting for growth. In discussing the experiments, we will abbreviate the "hungry" codon in lower case letters. For example, the construct with the sequence U UUC **aag** was subjected

Table 1. Slippery sequences and conditions of aminoacyl-tRNA limitation used to promote frameshifting

Frameshift sequence ^a	Aminoacyl-tRNA ^b	Method of starvation ^c	Relative growth rate ^d	
			CP78	CP79
U UUC AAG	Lys	Lysine hydroxamate (40 µg/ml)	0.22	0.21
U UUC UUA	Leu	Leucine methyl ester (10 µg/ml)	0.14	0.13
U UUC AUA	Ile	Isoleucine-hydroxamate (72 µg/ml)	0.29	0.30
U UUC AUA	His, then Ile	CP medium lacking His, followed by isoleucine hydroxamate	–	0.30

^aThe relevant portion of each frameshift sequence tested is shown

^bThe amino acid encoded after the slippery UUUC quadruplet is indicated

^cInhibitor used to limit the supply of the indicated amino acid; the concentration of hydroxamate (amino acid hydroxamates specifically inhibit corresponding aminoacyl-tRNA synthetases) or amino acid derivative used is indicated in *parentheses*. The strains used are leucine auxotrophs, and utilize leucine-methyl-ester for growth at a rate directly related to its concentration, presumably due to slow uptake and/or hydrolysis

^dThe growth rates are expressed as doubling times (relative to unstarved or uninhibited cells) of CP78 (*relA*⁺) and CP79 (*relA*) under the indicated starvation regimes

to lysine-tRNA limitation, that with sequence U UUC **aua** was subjected to isoleucine-tRNA limitation, and so forth.

Here, we report on the timecourse of frameshifting in response to stalling the ribosome at lysine, leucine, and isoleucine codons in isogenic wild-type and *relA* mutant cells. The results (Fig. 1) are presented in the form of differential plots of enzyme synthesis versus increase in protein. This is the classical mode of representation of induced enzyme synthesis introduced by Monod and his associates at the Pasteur Institute (Monod 1958; reviewed by Pardee 1962), in which the slope is proportional to the fraction of total protein synthesis represented by active β -galactosidase.

Response of *relA*⁺ and *relA* cells to amino acid limitation

It can be seen that limitation for each aminoacyl-tRNA species promoted frameshifting to one extent or another at the corresponding hungry codons in both genotypes. In the *relA*⁺ background the increase in β -galactosidase synthesis was essentially constant from the start, and exhibited a differential rate a few times greater than the control, unstarved cells. In the *relA* mutant strain, the differential rate was increased considerably more from the start in the cases of lysyl-tRNA limitation (Fig. 1B) and leucine limitation (Fig. 1D). These results confirm older reports (Gallant and Foley 1980; Weiss and

Fig. 1A–F. Leftward frame-shifting on three reporter constructs in the isogenic *relA*⁺ and *relA* strains. All data are reported as differential plots of the increase in β -galactosidase activity versus the increase in total protein after induction (Monod 1958; Pardee 1962). In each case, experiments with the wild-type *relA*⁺ strain CP78 are shown on the left (A, C, and E), while corresponding experiments with the *relA* mutant strain CP79 are shown on the right (B, D, and F). The Y-axis is calibrated in units of β -galactosidase activity as defined in Materials and methods. In each case, the open diamonds show the data for control, uninhibited cells, whereas the open squares represent the results for cells subjected to the specified limitation regime. A, B Shifty sequence U UUC **aag**; lysyl-tRNA limitation elicited by lysine-hydroxamate (40 mg/l). C, D Shifty sequence U UUC **uua**; leucine limitation elicited by resuspending washed cells in fresh medium containing leucine methyl ester (10 mg/l) in place of leucine; the control cultures were resuspended in medium containing leucine (100 mg/l). E, F Shifty sequence U UUC **aua**; isoleucyl-tRNA limitation elicited by isoleucine-hydroxamate (72 mg/l). The data in A and B come from two different experiments performed 2 years apart by two different authors. The other panels show representative single experiments. The insert in F shows a close-up of the first points in order to clarify the lag period

Gallant 1983) that starvation-promoted frameshifting is partly dependent on an inactivated *relA* gene. However, it is worth noting that the dependence is by no means absolute: frameshifting clearly increased in the *relA*⁺ cells as well.

In the case of isoleucyl-tRNA limitation, the response of the *relA* strain showed more complex kinetics (Fig. 1F). There was little increase at first (see the insert in Fig. 1F), but then an enormous, progressive increase, so that by the second doubling of partly starved growth the differential rate was 60 times greater than in the unstarved control cells. Thus, the difference between the response of *relA*⁺ and *relA* cells is greatest in this case (4-fold versus 60-fold stimulation) but only after about a doubling of isoleucine-tRNA-limited growth. A similar gradual increase in the rate of leftward frameshifting on the related sequence UUU *aua* has also been demonstrated during isoleucyl-tRNA limitation of *relA* mutant cells (Gallant et al. 2000). We note that these kinetics are independent of the use of hydroxamates to inhibit the aminoacyl-tRNA synthetase. A very similar gradual rise in frameshifting, specific to the *relA* strain, was observed when we limited for the amino acid isoleucine (data not shown).

Effect of prior starvation for histidine on frameshifting in *relA* cells limited for isoleucyl-tRNA

The growth lag before maximum frameshifting in isoleucyl-tRNA limited *relA* cells suggests that some factor which exacerbates frameshifting accumulates gradually. We therefore asked whether prestarvation for a different amino acid entirely would lead to accumulation of such a factor. To do so, we subjected the cells, which are auxotrophic for histidine, to 80 min of histidine starvation. Histidine was then restored with or without simultaneous addition of isoleucine-hydroxamate (ILHX). As Fig. 2 shows, the period of histidine starvation markedly reduced the growth lag preceding ILHX-induced frameshifting. We conclude that the factor which potentiates the left frameshifting event at a hungry isoleucine codon can accumulate during pre-starvation for histidine (and perhaps any other amino acid) as well as for isoleucyl-tRNA.

The *tgt* locus has no effect on the kinetics of frameshifting

The growth lag kinetics typify frameshifted reading starting with histidine tRNA, reading the C *au* triplet overlapping the hungry codon; and we have also observed similar kinetics for frameshifted reading by tyrosine tRNA, reading an overlapping U *au* triplet in the related shifty sequence UUUU *aua* (Gallant et al. 2000). These two tRNAs are among the four species which contain queosine at the wobble position, nucleotide 34 (Sprinzl et al. 1996). One possible explanation of the

Fig. 2. Effect of prestarvation for histidine on ILHX-induced frameshifting. The experiments were performed with CP79 (*relA*) carrying the U UUC *aua* left-frameshift reporter. Control cells were induced as in Fig. 1, with one uninhibited subculture (open circles) and one subjected to ILHX (72 μg/ml) inhibition (filled circles). A portion of the culture was centrifuged, washed, resuspended in medium lacking histidine, and starved of histidine for 80 min. Histidine was then added back, and one subculture was induced directly (open squares) while the other was induced and simultaneously subjected to ILHX (72 μg/ml) inhibition (filled squares). The insert shows a close-up of the early points for the two ILHX-inhibited cultures, to emphasize the shortening of the lag elicited by prior histidine starvation

results might be that starved *relA* cells gradually accumulate undermodified, queosine-deficient tRNAs which may be prone to frameshifted reading. As a general test of this hypothesis, we examined the effect on frameshifting of a mutant allele of the *tgt* gene, which results in a queosine deficiency in all four tRNAs (Frey et al. 1989; Reuter et al. 1994).

Figure 3 rules out the hypothesis, showing that frameshifting efficiency on our reporter is identical in *tgt*⁺ and *tgt* mutant cells. It follows that frameshifting at our site is not sensitive to the queosine modification of histidine or tyrosine tRNA. Bierley and coworkers have previously shown that the queosine modification has little effect on leftward frameshifting at certain viral programmed frameshift sequences in *E. coli* (Bierley et al. 1997).

Discussion

Our results suggest that (at least) two components contribute to the stimulation of left frameshifting brought about by aminoacyl-tRNA limitation. The first, which is independent of the *relA* function, is common to *relA*⁺ and *relA* mutant cells. We presume that this effect is a direct and immediate result of the decreased level of aminoacyl-tRNA and consequent ribosome pausing at hungry codons cognate to that aminoacyl-tRNA.

The second component is a further stimulation of frameshifting observed in the *relA* genotype specifically. In the cases of limitation for isoleucine-tRNA, this component is distinguished by the strikingly slow kinetics with which it develops (Figs. 1F and 2). The slow

Fig. 3. Lack of effect of *tgt* on leftward frameshifting. Exponentially growing cells of SJ1502 (*tgt*⁺, filled circles) and SJ1505 (*tgt*⁻, open circles) carrying the U UUC AUA left-frameshift reporter were induced and samples removed over a period of about two generations for assay of β -galactosidase and protein

kinetics strongly suggest that this additional, *relA*-specific component is an indirect effect of starvation. This, and the pre-starvation experiment shown in Fig. 2, suggests that partly (or completely) starved *relA* cells accumulate some factor which increases shiftiness at the hungry codon. What could this factor be?

It was shown long ago that *relA* mutants accumulate hypomodified tRNAs during partial or complete amino acid starvation (Kitchingman and Fournier 1975, 1977; Fournier et al. 1976).

In an important recent study, Björk's group has demonstrated that several different tRNA modifications in numerous isoacceptors play an important role in reading frame maintenance (Urbonavicius et al. 2001). Therefore, an undermodified tRNA which participates in the frameshift event may well be the culprit. There are three ways in which tRNAs participate. Peptidyl-tRNA in the ribosome's P-site must slip left to permit frameshifted reading of the triplet overlapping the hungry codon (Kolor et al. 1993; Barak et al. 1996; Farabaugh 1996, 1997; Gallant et al. 2000). Second, an aminoacyl-tRNA reads the frameshifted triplet overlapping the hungry codon from the left side (Kolor et al. 1993; Barak et al. 1996; Gallant and Lindsley 1996). Third, we should bear in mind that the aminoacyl-tRNA cognate to the hungry codon plays a role (reduced by virtue of its limited acylation) as competitor with the frameshifting aminoacyl-tRNA.

The aforementioned study by Urbonavicius et al. (2001) showed that undermodified forms of several tRNAs are prone to slip rightward as peptidyl-tRNA in the ribosome's P-site, and comparable tendencies toward leftward slippage would seem quite likely. Unfortunately, this straightforward explanation cannot account for the kinetics we have observed. All three of the constructs described here have the same sequence (U UUC) upstream of the hungry codon, and therefore all three involve peptidyl-tRNA slippage by phenylalanine tRNA. Yet one of these constructs shows gradual

kinetics in *relA* cells (Figs. 1F and 2) while two do not (Fig. 1B and D).

The difference between these two pairs of experiments lies in the hungry codon and in the regime of limitation for an amino acid or aminoacyl-tRNA. The tRNA which initiates the frameshift may therefore be the key variable. If this is correct, it follows that undermodified forms of histidine-tRNA (Fig. 1F) and tyrosine-tRNA (Gallant et al. 2000) are particularly prone to read an overlapping codon, but this is not true of the glutamine-tRNA and the leucine (C uu)-tRNA isoacceptors responsible for the starvation-promoted frameshifting on the sequences U UUC aag (Fig. 1B) and U UUC uua (Fig. 1D), where frameshifting does not increase with time in the *relA* strain. As to what the modification in question is, Fig. 3 demonstrates only that queosine modification is not it.

Another possibility is that the *relA* deficit in tRNA modification is indeed at issue, but that the tRNA involved is the limiting species which normally reads the hungry codon. Certain tRNA modifications, particularly those at positions 34, 37, and 38–40, have been shown to affect the efficiency of translation (reviewed in Björk 1992; Curran 1998; Urbonavicius et al. 2001). Suppose that certain undermodified tRNAs (such as tRNA-isoleucine-2, which reads AUA) show reduced translational efficiency. Then, as *relA* cells grow under limitation for any amino acid or aminoacyl-tRNA, undermodified forms will come to make up a larger and larger fraction of the tRNA population in question, and ribosomes will stall for longer and longer periods at codons calling for the tRNA, thus progressively increasing the probability of frameshifting.

Further experiments will be needed to distinguish this kind of model from one involving tRNAs that participate directly in the frameshift event. Since tRNAs carry modifications at between 6 and 10 different positions, it would be pointless at this stage to speculate on which modifications might be involved. In any event, it appears that some aspect of the starved *relA* syndrome contributes gradually and indirectly to the probability of leftward frameshifting, perhaps by affecting the modification status of tRNAs. The molecular basis of this indirect effect remains to be elucidated, but it need not reflect noncognate tRNA recognition, as we once postulated (Kurland and Gallant 1986).

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