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NOTES

Does Disparate Occurrence of Autoregulatory Programmed Frameshifting in Decoding the Release Factor 2 Gene Reflect an Ancient Origin with Loss in Independent Lineages?

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In *Escherichia coli* an autoregulatory mechanism of programmed ribosomal frameshifting governs the level of polypeptide chain release factor 2. From an analysis of 20 sequences of genes encoding release factor 2, we infer that this frameshift mechanism was present in a common ancestor of a large group of bacteria and has subsequently been lost in three independent lineages.

The advent of complete genome sequences provides the opportunity to assess the conservation of programmed ribosomal frameshifting in the expression of particular genes. The sequences also permit deductions about single or multiple origins of frameshift cassettes and the degree of conservation of the signals involved. A second reason to examine published sequences for a particular frameshift cassette is to highlight the need for caution in assigning genes by comparative methods. Gene assignments in some genome-sequencing papers have been based exclusively on homology to a single open reading frame (ORF), while other investigators have been mindful of the fact that the synthesis of some proteins involves a pro-

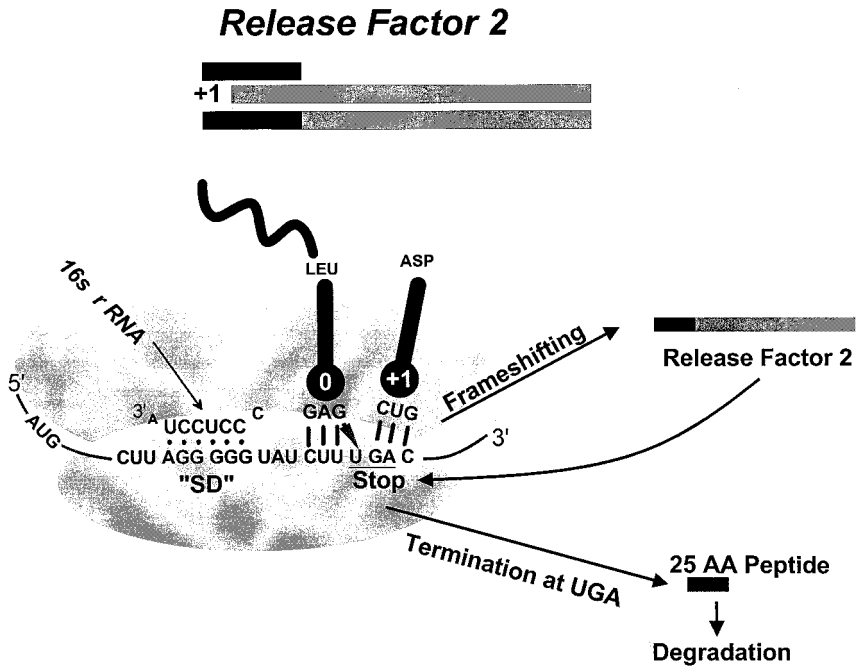

FIG. 1. Current model for RF2 frameshifting. See text for details.

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A. aeolicus      ~~~~~~MMMV ELKGKVEELR KRLEDVKKIL SPEKLESELK ELDQKMSEPN
B. firmus        ~~~~~~~~~~ ~~~~~~~~MA KRLTDFRGSL DLEAKQERMA ELDEFMTAPD
B. subtilis      ~~~~~~MELS EIRAELENMA SRLADFRGSL DLESKEARIA ELDEQMADPE
B. burgdorferi   ~~~~~~~~~~ ~MKEKINTLL KHAEDIWRKL DKNEIQAKIE KYEKEINQKN
C. trachomatis   ~~~~~~~MHE NFDKRLELLL EGLALTRRSL DPEGKENELK ELEQQAVQDG
C. acetobut.     ~MIFLLIKIE EAASLIKEIE IKAREMRASL DFDKSRSRID ELERDMQEPD
D. radiodurans   ~~~~~~~~~~ ~~~~~MQELL EKLASLREYL DIPGKTRRLN ELDRELSDPD
E. coli          ~~~~~MFEIN PVNNRIQDLT ERSDVLRGYL DYDAKKERLE EVNAELEQPD
H. influenzae    ~~~~~MFEIN PVKNKIIDLS DRTSVLRGYL DFDAKVERLE EVNGELEQPD
H. pylori        ~~~~~~VDNY TYSELLKSLQ NKCDNIALII KPEKIKQELE RIEKEQEDPN
N. gonorrhoeae   ~~~MEAEVIN QLNNTLNDLE KRSEDIRVYM DYQGKKDRLE EVIGLSEDPE
M. leprae        ~~~~~~~MEP DRQTDIAALD STLTTVERVL DVEGLRTRIE KLEHEASDPK
M. tuberculosis MPVTLAAVDP DRQADIAALD CTLTTVERVL DVEGLRSRIE KLEHEASDPH
P. aeruginosa    ~~~~~~MEIN PILNSIKDLS ERTQTIRGYL DYDQKHDRLV EVNRELEDPN
S. typhimurium   ~~~~~MFEIN PVNNRIQDLT ERTNVLRGYL DYDAKKERLE EVNAELEQPD
S. coelicolor    ~~~~~~MAVV DVSEELKSLS STMESIEAVL DLDRLRADIA VLEEQAAAPS
S. pneumoniae    ~~~~~~MDIS VIRQKIDANR EKLASFRGSL DLEGLEEEIA ILENKMTEPD
S. pyogenes      ~~~~~~MEVA EIRQKIVENK EKLTSFRRSL DLDRLEEEIA LLENHMTEPD
Synechocystis    ~~~~MITELT DLKRNLELIS SRLGQTQDYL DLPGLKAKVQ DLEQCAAQPD
T. pallidum      ~~~~~~MEIE EFGRQITALE ARVQEVWGSL DVAAYEARIA TLEAAAAAPD
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FIG. 2. Alignment of the N-terminal end of RF2. The last preshift and first postshift amino acids are boldfaced. For the sources of the sequences, see the reference for each organism, as follows: *A. aeolicus* (38), *Bacillus firmus* (4), *B. subtilis* (33), *Borrelia burgdorferi* (14), *Chlamydia trachomatis* (37), *C. acetobutylicum* (15), *Deinococcus radiodurans* (15a), *E. coli* (8), *Haemophilus influenzae* (13), *Helicobacter pylori* (39), *Neisseria gonorrhoeae* (31), *Mycobacterium tuberculosis* (27), *Mycobacterium leprae* (12), *Pseudomonas aeruginosa* (30), *Salmonella typhimurium* (19), *S. coelicolor* (25), *Streptococcus pneumoniae* (15a), *Streptococcus pyogenes* (32), *Synechocystis* sp. strain PCC6803 (17), and *Treponema pallidum* (38).

grammed ribosomal frameshift event to link the information from two ORFs.

Expression of the *Escherichia coli* release factor 2 (RF2) gene, *prfB*, requires ribosomes at codon 25, CUU, to shift to the +1 frame, which encodes the main part of the protein (7, 8, 18) (Fig. 1). Codon 26 in the initiating frame is a UGA stop codon. RF2 mediates release at UGA, and in the presence of excess RF2, a high proportion of ribosomes terminate at codon 26 and only a small proportion shift to the +1 frame. The released 25-amino-acid peptide is degraded, and little full-length active RF2 is synthesized. However, when there is a deficit of RF2, the UGA, and pertinently its 1st base, U, is temporarily free. This U forms the 3rd base of a +1-frame UUU codon with which peptidyl $tRNA^{Leu}$ pairs following disengagement from the 0-frame CUU (40). This re-pairing involves first-position wobble pairing.

The RF2 frameshift site is conserved in a large number of distantly related bacteria. The nucleotide sequence of the region that signals programmed frameshifting in the RF2 gene in *Bacillus subtilis* is strikingly similar to that of its *E. coli* counterpart (26), but in *Streptomyces coelicolor* frameshifting does not seem to be involved (25). With the recent increase in available genome sequence information, we collected 20 RF2 sequences from different bacteria. This was achieved with the help of the Entrez Browser (12a), the Blast (2) server at the Institute for Genome Research (15a), the Gonococcal and Streptococcal Genome Projects (31, 32), the *Pseudomonas* Genome Project (30), and the *Chlamydia* Genome Project (37). In addition, we obtained the *Aquifex aeolicus* RF2 sequence from R. Swanson at Diversa Corp. All sequences were aligned with the PILEUP program of the Genetics Computer Group package and manually searched for possible frameshift sites. Particular caution was taken in the alignment of the first part of the RF2 amino acid sequences (Fig. 2), and homology before the potential frameshift sites was examined carefully to determine whether a frameshift was likely to take place or not. All sequences allowed unambiguous detection of the absence or presence of a frameshift site. Nonframeshifters lacked an in-frame stop codon and had continuous sequence similarity to the coding frames of other RF2 sequences both before and after their frameshift sites. Sequences with the frameshift site all had possible start sites only in the 0 frame upstream of the shift site and had a UGA stop codon in the 0 frame at a position corresponding to the beginning of the +1-frame homologous sequences. Their products were also homologous RF2s. In no case did we detect any homologs of RF2 other than RF1 and occasionally RF-H (release factor homolog); therefore, these organisms are likely to harbor only one *prfB* gene.

All RF2 frameshift sites identified in the DNA sequences have a conserved CTTTGAC motif (Fig. 3). The position of the frameshift site is the same in all organisms, and even in some organisms that do not shift, the leucine-aspartate codons are conserved, which suggests that these amino acids are structurally important in the RF2 molecule. Maybe this provides additional selection pressure, together with the autoregulatory

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Sequences with shift sites:

      -SD-- spacer   UGA
ACTTTAGGGGG  TCT  CTTTGACTTA Bacillus firmus
ACTTTAGGGGG  TCT  CTTTGACCTC Bacillus subtilis
ACATTTGGAGG  AAG  CTTTGACAAA Borrelia burgdorferi
TAACTCGGAGG  TCT  CTTTGACCCG Chlamydia trachomatis
AAATGAGGGCT  TCT  CTTTGACTTC Clostr. acetobutylicum
CGCTCCGGGAG  TAC  CTTTGACATT Deinococcus radiodurans
TTCTTAGGGGG  TAT  CTTTGACTAC Escherichia coli
TGCTTCGGGGG  TAT  CTTTGACTTC Haemophilus influenzae
CCATTCGGGGG  TAT  CTTTGACTAC Pseudomonas aeruginosa
TTCTTAGGGGG  TAT  CTTTGACTAC Salmonella typhimurium
CTTTCAGGGGG  TCT  CTTTGACCTC Streptococcus pneumoniae
GCTTCAGGAGG  TCT  CTTTGACTTA Streptococcus pyogenes
AAACCCAGGAC  TAT  CTTTGACCTG Synechocystis PCC6803
AGGTATGGGGG  AGT  CTTTGACGTT Treponema pallidum
                ...Leu***
                     Asp...

GAAACGGGCCG  CTA  CTCTGACACA E. coli prfH

Sequences without shift sites:

ATATCGCTTTA  ATC  ATC AAGCCT  Helicobacter pylori
CGGTGGAGCGG  GTG  CTC GACGTG  Mycobacterium leprae
CGGTGGAGCGG  GTG  CTC GACGTC  Mycobacterium tuberculosis
ACATCCGCGTT  TAT  ATG GATTAT  Neisseria gonorrhoeae
CGATCGAGGCC  GTC  CTG GACCTC  Streptomyces coelicolor
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FIG. 3. Alignment of RF2 frameshifting sites and the nonfunctional similar site in *E. coli prfH*. Sequences were obtained from the sources cited in the legend to Fig. 2.

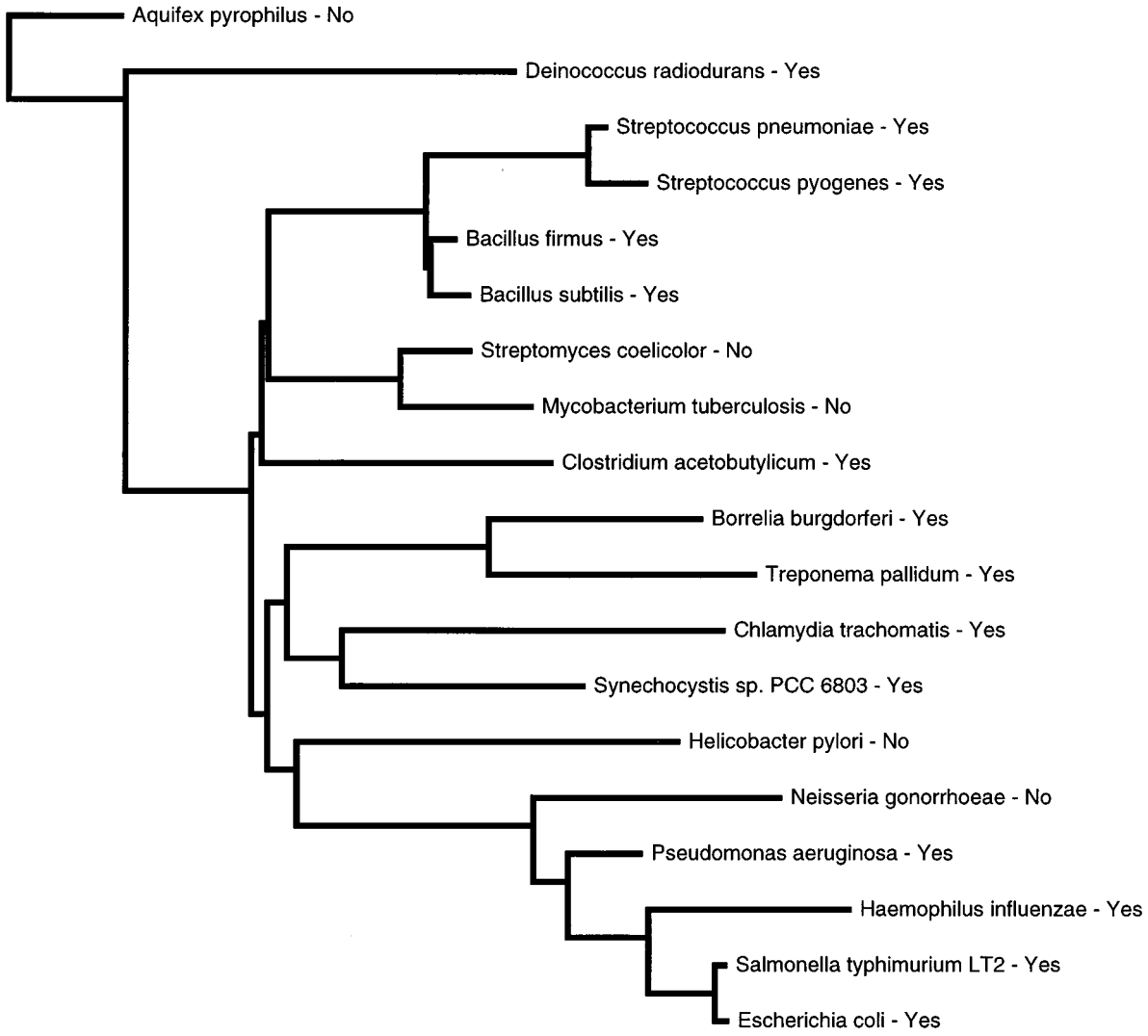

FIG. 4. Phylogenetic tree based on 16S rRNA obtained from the Ribosomal Database Project. Because the 16S rRNA sequence from *A. aeolicus* was unavailable, the sequence of *Aquifex pyrophilus* was used. Yes, RF2 frameshift site present; No, RF2 frameshift site absent.

mechanism, to keep the sequence in the organisms that do frameshift. The stop codon is always UGA, which allows RF2 autoregulation. The codon preceding the shift is always CUU. In *E. coli*, replacement of CUU by other codons which permit their decoding tRNA to re-pair with the overlapping +1-frame codon allows frameshifting (9, 40), although CUU itself causes the most efficient frameshifting (9). As in other systems, rather weak preshift pairing and relatively strong postshift pairing is important for RF2 frameshifting (9). The identities of the two carboxy-terminal amino acids of the nascent chain influence termination in *E. coli* (3, 5). Although there seems to be a preference for tyrosine, valine, or serine as the amino acid preceding leucine, this is likely to reflect demands on the RF2 structure rather than effects on termination. In test constructs, when the UGA stop codon is replaced with a sense codon with U as its 1st base, the potential for pairing with the overlapping +1-frame UUU is retained. However, the level of frameshifting is substantially reduced. The flanking stop codon is important but not essential for the frameshifting (11, 36, 40, 42), although it is crucial for autoregulation.

The 1st codon in the new frame is in all cases a GAC aspartate codon. The alternative aspartate codon, GAT, is not found, presumably because the UGA stop codon is most efficient when followed by a C. Following several early studies (see references 6 and 34), numerous reports have shown that the identity of the base following a triplet stop codon substantially influences the efficiency of termination. The termination codon may effectively be a quadruplet (28, 29). In *E. coli*, UGAC is a comparatively poor terminator, and it is probably not coincidental that the UGA at codon 26 in the gene for RF2 is followed by C (23, 28). Since there is competition between frameshifting and termination, as well as in-frame readthrough (1, 10), having a poor terminator permits more efficient frameshifting.

In all cases the shift site is preceded by a G-rich sequence at a variable distance from the shift site. This element is important for a Shine-Dalgarno-like interaction, which involves translocating, rather than initiating, ribosomes (10, 35, 40–42). Pairing between 16S rRNA of ribosomes and a Shine-Dalgarno sequence 3 bases 5′ of the shift site directly stimulates +1 frameshifting. Mutagenesis experiments have shown that precise positioning of the Shine-Dalgarno sequence is required

(40) and that spacing between the Shine-Dalgarno sequence and the shift site influences the directionality of shifting (20, 21). In *E. coli* this spacing has to be 3 nucleotides. This spacing is conserved in most of the organisms analyzed, although, interestingly, *Clostridium acetobutylicum* and *Synechocystis* sp. strain PCC6803 seem to be exceptions to this rule.

Several bacterial lineages have independently lost the RF2 frameshift site. With the help of the Ribosomal Database Project web site (22), a phylogenetic tree based on the 16S rRNA of these organisms was constructed (Fig. 4). The phylogenetic tree of bacteria with the RF2 frameshift site suggests that this autoregulatory element was acquired by an early ancestor of a large group of present-day bacteria ranging from green nonsulfur bacteria and cyanobacteria to purple and gram-positive bacteria. Then the frameshift mechanism seems to have been independently lost in at least three branches of the bacterial phylogenetic tree, leading to its absence in mycobacteria, *Streptomyces*, *Neisseria*, and *Helicobacter*. It will be interesting to see how the RF2 levels are regulated in these organisms.

We searched for the sequence GGGGGNNNCTTTGAC at other locations in the genome of *E. coli*. Several sequences with resemblance to this motif were found, but none was found in the productive reading frame within a coding region. There is one gene in *E. coli*, *prfH*, that encodes a protein homologous to RF2. In the beginning of this gene there is a sequence with some similarity to the frameshift site of *prfB* (Fig. 3). We tested this sequence for its frameshifting ability in vivo by inserting the sequence between *gst* and *lacZ*, with *gst* being fused in the 0 frame and *lacZ* being fused in the +1 frame. No frameshifting activity could be detected by assaying for β-galactosidase (data not shown).

Many cases of programmed frameshifting are known, or suspected, in the decoding of viral genes and transposable elements, and a small number are known for cellular gene decoding. Very little is yet known about the phylogeny of frameshifting cassettes, but *dnaX* frameshifting in widely divergent eubacteria (21, 24, 43) is being compared, as is antizyme frameshifting in *Drosophila* and humans (16).

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