RNA (2002), *8*:16–28. Cambridge University Press. Printed in the USA.

DOI: 10.1017.S1355838201012080

Influence of the stacking potential of the base 3′ of tandem shift codons on –1 ribosomal frameshifting used for gene expression

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ABSTRACT

Translating ribosomes can shift reading frame at specific sites with high efficiency for gene expression purposes. The most common type of shift to the –1 frame involves a tandem realignment of two anticodons from pairing with mRNA sequence of the form X XXY YYZ to XXX YYY Z where the spaces indicate the reading frame. The predominant –1 shift site of this type in eubacteria is A AAA AAG. The present work shows that in *Escherichia coli* the identity of the 6 nt 3′ of this sequence can be responsible for a 14-fold variation in frameshift frequency. The first 3′ nucleotide has the primary effect, with, in order of decreasing efficiency, U > C > A > G. This effect is independent of other stimulators of frameshifting. It is detected with other X XXA AAG sequences, but not with several other heptameric –1 shift sites. Pairing of *E. coli* tRNA^{Lys} with AAG is especially weak at the third codon position. We propose that strong stacking of purines 3′ of AAG stabilizes pairing of tRNA^{Lys}, diminishing the chance of codon:anticodon dissociation that is a prerequisite for the realignment involved in frameshifting.

Keywords: –1 frameshifting; 3′ context effect; codon–anticodon interaction; *Escherichia coli*; recoding; tRNA^{Lys}; X XXY YYZ frameshift motifs

INTRODUCTION

Programmed recoding events such as stop codon readthrough or frameshifting serve a regulatory role or allow the synthesis of unexpected but biologically important protein species. Among these, −1 frameshifting was found to be required for the expression of a limited number of genes in viruses, for example, to produce the Gag-Pol polyprotein of retroviruses, as well as to produce the transposase of prokaryotic insertion sequences (Chandler & Fayet, 1993; Farabaugh, 1997; Atkins et al., 2001; Brierley & Pennell, 2001). In most of the known cases of −1 programmed frameshifting, the ribosome shifts one nucleotide backward on heptameric sequences of the type X XXY YYZ, where X, Y, and Z can be different or identical nucleotides. The codons XXY and YYZ dissociate from the cognate tRNA anticodons and the mRNA slips by one base and repairs to the anticodons via the codons XXX and YYY, thereby causing a shift to the −1 frame (Jacks et al., 1988). Although eukaryotic and prokaryotic translational machineries respond to the same type of slippery motifs, they do not respond in the same way to individual heptamers (Weiss et al., 1989; Brierley et al., 1992; Tsuchihashi & Brown, 1992). Beside obvious overall structural and functional similarities of their components, they differ in many aspects. Among these, some subtle structural features of the decoding tRNAs probably determine the degree of shiftiness of each motif in a given organism.

Natural programmed frameshift regions induce rephasing at a higher frequency than isolated heptameric motifs because they contain stimulatory sequences. Here again, similarities and differences are found between eukaryotic and prokaryotic signals. Just one type of stimulatory element 5′ of heptameric −1 programmed shift sites is known and it is specific of eubacteria. The 3′ end of 16S rRNA of translating ribosomes scans mRNA for potential complementarity and its pairing with a Shine–Dalgarno sequence 9–14 bases 5′ of a −1 shift site stimulates −1 frameshifting at that site (Larsen et al., 1994; Mejlhede et al., 1999). On the 3′ side of heptameric motifs, mRNA structured elements were of-

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ten found to be efficient stimulators of frameshifting. In retroviruses and coronaviruses, the heptameric motif is generally followed, 5 to 9 nt downstream, by a pseudoknot (Brierley et al., 1989; ten Dam et al., 1990) whereas in barley yellow dwarf virus, both a proximal and a much more distant stimulatory element operate (Paul et al., 2001). In bacteria, the structural element is generally a stem-loop located 5 to 8 nt downstream (Larsen et al., 1997; Rettberg et al., 1999).

In addition to the effect on programmed −1 frameshifting of the downstream elements presented above, other types of 3′ effects have been found for normal decoding as well as for different recoding events. Even though they are of diverse, and not fully understood, origins, we will call these collectively "immediate 3′ context effects" because they implicate only 1 to a few nucleotides following a codon or a recoding site. A series of observations suggests that the 3′ context of sense and stop codons has an impact on their translation (Looman et al., 1987; Buckingham, 1994; Irwin et al., 1995). For example, suppression of stop codons (Bossi, 1983; Miller & Albertini, 1983; Pedersen & Curran, 1991) and termination at normal gene terminators (Poole et al., 1995; Tate & Mannering, 1996; Pavlov et al., 1998) were shown to be sensitive to the identity of the nucleotide immediately 3′. In the former studies, which are the most relevant to our work, the proposed explanation is that the 3′ nucleotide stacks on the codon:anticodon helix and can thus modulate the stability of the tRNA:mRNA interaction (Ayer & Yarus, 1986; Stormo et al., 1986; Pedersen & Curran, 1991).

Immediate 3′ nucleotides have also been found to affect codon redefinition where standard amino acids are encoded by what are stop codons in standard decoding. Most of these range from a few nucleotides (Skuzeski et al., 1991; Wills et al., 1994) to only 1 nt (Li & Rice, 1993). An effect of the 3′ adjacent base on +1 frameshifting has previously been studied in yeast (Pande et al., 1995). In this type of +1 frameshifting where re-pairing is involved, the 3′ +1 frame base is part of the "re-pairing" codon. A bacterial counterpart is provided by the very efficient RF2 +1 frameshifting signal (Weiss et al., 1988), which has also been used to analyze 3′ context effect on sense and nonsense codons (Pedersen & Curran, 1991; Curran et al., 1995; Major et al., 1996).

In the case of −1 frameshifting, a limited number of sometimes contradictory reports addressed the problem of the effect of the 3′ immediate context (i.e., the 5 to 9 nt between the shift site and the stimulatory sequence, if present) on the efficiency of recoding. A recent study (Kim et al., 2001) showed that, in an in vitro eukaryotic translation system, frameshifting is moderately (twofold variation) influenced by the nature of the 6 nt downstream of the U UUU UUA and A AAA AAC motifs of HIV-1 and HTLV-2; however, only two different downstream sequences were tested. A stop codon was found to be an activator when located immediately 3′ to a G GGG site present in synthetic frameshift windows (Weiss et al., 1990) or to the A-AAA motif of potato virus M (Gramstat et al., 1994). In other situations, like in Rous Sarcoma Virus (Jacks et al., 1988) and Infectious Bronchitis Virus (Brierley et al., 1992, 1997), an adjacent stop codon appeared to have no effect on frameshifting. In contrast with these reports, Tate's group found that a stop codon at the same position has a negative effect on frameshifting on a U-UUU-UUA motif both in *Escherichia coli* (Horsfield et al., 1995) and eukaryotes (McKinney, 2001).

The lack of a clear understanding of the incidence of the shift site immediate 3′ context on −1 frameshifting lead us to carry out a systematic investigation in *E. coli*. For that, we choose the −1 frameshift region of insertion sequence IS*911*. This transposable element of the IS*3* family (Chandler & Fayet, 1993) expresses its transposase via −1 programmed frameshifting on an A AAA AAG motif (Polard et al., 1991; Rettberg et al., 1999). Our approach was to mutagenize the 6 nt following the heptameric motif. We found that the modification of these nucleotides modulates the level of frameshifting over a wide range.

RESULTS

Mutagenesis strategy

In IS*911*, frameshifting occurs between two consecutive genes, orfA and orfB, that overlap by 94 nt. It generates a fusion protein, containing most of the OrfA and OrfB products, that is essential for transposition (Polard et al., 1991). The region necessary to promote maximal frameshifting is about 100 nt long (Fig. 1). The A AAA AAG tandem shift site is preceded by an AUU codon, used as start codon for OrfB synthesis, and is surrounded by two stimulators, a Shine-Dalgarno sequence located 11 nt upstream and a stem-loop structure with a three-way junction situated 6 nt downstream (Polard et al., 1991; Rettberg et al., 1999). The short region called spacer 2 (SP2) that separates the motif and the stem-loop (Fig. 1) was randomly mutagenized, using overlapping and degenerate oligonucleotides (see Materials and Methods), and the mutated IS*911* frameshift region was introduced between the *g10* and *lacZ* reporter genes of plasmid pOFX302 (Rettberg et al., 1999). The *g10* and *lacZ* genes are fused to the end of the IS*911 orfA* gene and to the beginning of the IS*911 orfB* gene, respectively. Therefore, *lacZ* is in the −1 phase relative to *g10*. The production of the G10-A′:B′-LacZ fusion protein resulting from −1 frameshifting is measured either after radiolabeling and electrophoresis on an acrylamide gel or by β-galactosidase assay, provided the AUU start codon has been mutated to ACU to prevent synthesis of the B′-LacZ species. A control construct, in which the fusion protein is pro-

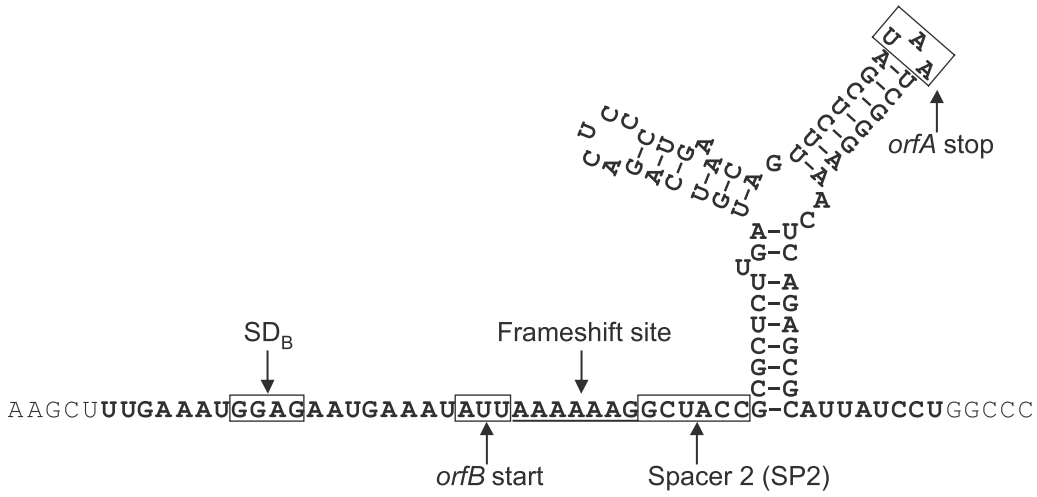


FIGURE 1. The IS*911* frameshift region. The 100-nt IS*911* segment shown (in bold) is flanked by sequences used for cloning. The first zero frame codon in this sequence is AGC (nt 2–4) and this OrfA frame terminates with the boxed UAA. When ribosomes shift to the −1 frame at A AAA AAG motif, translation extends beyond this sequence and in most constructs used terminates at the stop codon of lacZ. The 6 nt between the shift site and stem-loop structure form the spacer 2 region (SP2).

duced constitutively, was made by changing the A AAA AAG heptamer to the nonshifty A AGC GUG G octamer (plasmid pOFX302-361; Rettberg et al., 1999). The level of frameshifting of all the mutants is expressed as a percentage of the quantity of fusion protein obtained with this control construct.

Effect of six 3′ nucleotides—primary role of the first nucleotide

We obtained 158 mutants by the above mutagenesis and the quantity of fusion protein was measured for each one by the pulse labeling assay (Table 1). In addition, 16 mutants were constructed in which only the first 2 nt of spacer 2 were modified (Table 2). The analysis of the data in Table 1 revealed that changes in SP2 cause an important variation of the rephasing level. The difference between the highest (mutant 158 UAGUGC, 62.91%) and the lowest (mutant 1 GUAUGC, 4.38%) mutant is approximately 14-fold. The modification of only two positions (Table 2) also leads to significant differences, though not to the same extent. There is a 5-fold difference between the lowest (GUUACC 6.04%) and the highest (CCUACC 30.17%). Thus, although downstream of the shift site, the SP2 sequence modulates the level of frameshifting.

To try to find if there was a pattern, we sorted our collection of mutants according to their sequence in different ways: (1) sorting by sequence only (i.e., seeing if the identity of any of the six positions, taken singly or associated with one or two others, can explain the variations) and (2) sorting by codon (i.e., looking at the consequence of changes on the zero phase [nt 1-2-3 and 4-5-6] and −1 phase codons [G-1-2, 3-4-5, 6-G-C]). The only meaningful pattern we found was from sorting according to the first nucleotide of SP2. The average level of frameshifting is 37.90% for U as first nucleotide of SP2, 30.80% for C, 17.80% for A, and 11.80% for G (Table 1 and Fig. 2). However, the following nucleotides also play a role, as indicated by the fact that the distributions shown in Figure 2 are largely overlapping. If they do it generally in a subsidiary manner, their effect can nevertheless be of the same magnitude as the effect of changes at the first position. When mutants differing at positions 1, or 1 and 2, or 1, 2, and 3 of SP2 are compared (Tables 1 and 2), then the average rule U > C > A > G is generally verified but with minor deviations (e.g., some A-starting mutants are equal to, or slightly above, the corresponding C-starting mutants). Consequently, the difference between these constructs is very variable, ranging from none (e.g., mutants GGUACC and AGUACC in Table 2) to 14-fold as observed between mutants 1 (GUAUGC) and 158 (UAGUGC). If the comparison is now made between mutants from Table 1 having in common the first 2 or 3 nt of SP2, then the variation ranges from none (e.g., mutants 14 GUUCUC and 15 GUAUGG) to 4-fold (e.g. mutants 1 GUAUGC and 31 GUGCAA); it even goes up to 5.8-fold for UU-beginning mutants because of one particular mutant. Mutant 119 (UUGGUG) displays a remarkably low level of frameshifting for its class, 8.90%, especially when compared to the 40.30% of mutant 144 (UUGCUG), which differs by only 1 nt. Mutant 119 has been constructed and carefully verified twice and both constructs gave the same result. Though we cannot explain why it gives such a low level of frameshifting (it is the only mutant in which nt 4 to 6 of SP2 are GUG), it illustrates how only one nucleotide

TABLE 1. Spacer 2 variants.[a]

n°	spacer 2	%fs
1	G U A U G C	4.38
2	G U A A G C	4.98
3	G U A G A G	5.03
4	G U A C C G	5.25
5	G U C A G G	6.03
6	G U A G A U	7.05
7	G A A C C G	7.12
8	G A A C C U	7.68
9	G C C G C C	7.88
10	G U U A C G	7.99
11	G U U A C C	8.04
12	G U A G A A	8.66
13	G G C A C C	8.97
14	G U U C U C	9.12
15	G U A U G G	9.34
16	G C C U C C	10.46
17	**G C U A C C**	**10.50**
18	G C C C U G	10.94
19	G C G A U C	11.16
20	G G G G G C	11.40
21	G A G C C G	11.68
22	G U U U U U	12.41
23	G G G U G C	13.48
24	G A C C G A	13.64
25	G G C A A G	14.08
26	G A A A A A	14.31
27	G C C G U A	14.73
28	G A A C U A	14.89
29	G C C U U A	16.18
30	G A C C U A	16.41
31	G U G C A A	17.05
32	G G C U U A	19.67
33	G A C U U A	20.22
34	G C U A U G	20.48
35	G C C U G U	20.77
36	G C U A U A	22.33

11.79 ± 1.65

n°	spacer 2	%fs
37	A G A C G C	7.61
38	A G C C U G	8.40
39	A G G A G G	9.01
40	A C A G G C	9.61
41	A G U C C G	9.99
42	A G C G C G	10.66
43	A A C G G C	12.30
44	A G C U U G	12.95
45	A G A C A G	13.24
46	A G C C A G	13.55
47	A C C A G G	14.22
48	A U C C U G	14.34
49	A G G C U G	14.36
50	A U G G A U	15.00
51	A U A C A C	15.10
52	A C G G A U	16.35
53	A A C G A A	18.46
54	A A A A U C	18.71
55	A U C C U A	19.21
56	A C G A A U	19.53
57	A C A C U G	20.22
58	A C A G A C	20.44
59	A U C A A C	20.45
60	A A G C G A	21.98
61	A U U U G G	22.27
62	A A C U A C	22.34
63	A A C C U C	23.42
64	A U C A U G	23.69
65	A A C A A A	23.74
66	A U C U U G	24.01
67	A C C G U A	25.46
68	A C C A U C	27.21
69	A C C U U A	27.34
70	A C A C A A	30.12

17.80 ± 2.07

n°	spacer 2	%fs
71	C A A C C C	17.14
72	C C A A G C	17.83
73	C C C C C U	18.52
74	C G U G C C	19.18
75	C U U G G C	19.91
76	C U U A C C	20.62
77	C G A G C A	20.73
78	C G C A C A	21.57
79	C A G A G C	21.98
80	C G A C U G	23.02
81	C U A G C A	24.26
82	C C C C G C	25.40
83	C C U G C A	26.00
84	C U G G G U	26.54
85	C G U G G U	26.85
86	C A U C A C	27.16
87	C A G C A C	27.82
88	C C C A U C	27.84
89	C A U A C C	27.86
90	C U U C U G	28.80
91	C A G U G U	30.44
92	C C C A A C	30.68
93	C A A A U A	30.95
94	C U C U U G	31.09
95	C U C U U A	31.42
96	C C C U C G	32.19
97	C U C C U A	32.24
98	C A G A U C	32.61
99	C U G U U G	33.55
100	C C U G U U	34.06
101	C C G C U U	34.81
102	C C G C A U	34.89
103	C C G G U A	35.08
104	C A C C A U	35.28
105	C C U G G G	35.82
106	C C G C U A	36.00
107	C C C A A U	36.06
108	C C U U C U	36.14
109	C C G G U U	36.60
110	C C G C G A	37.09
111	C C G C A A	37.76
112	C C U U U A	39.59
113	C C G G C U	40.16
114	C A U U A C	40.51
115	C C G U C U	42.40
116	C C G U U C	42.93
117	C A U C A G	43.86
118	C C U G U A	45.15

30.80 ± 2.13

n°	spacer 2	%fs
119	U U G G U G	**8.88**
120	U A C C G C	21.35
121	U A C A C C	22.79
122	**U A A U U G**	**27.53**
123	U C A G G U	27.91
124	U U U U G G	28.95
125	U U C C C U	29.01
126	U A C G G C	29.16
127	U A C G U U	29.26
128	U A U A U G	29.50
129	U U G G A U	31.37
130	U U C G A A	31.68
131	U U C A A A	32.81
132	**U A A C U G**	**32.99**
133	U G G C U A	34.70
134	U U A A U A	34.75
135	U A U A U C	35.07
136	U U G U G G	35.50
137	**U A G A G A**	**36.45**
138	U G U C A G	36.78
139	U U C G A C	36.98
140	U U U C A C	38.01
141	U C G A G A	38.16
142	**U A A A A U**	**39.40**
143	U U A U U U	40.23
144	U U G C U G	40.27
145	**U A G C C A**	**40.53**
146	**U A A U G G**	**40.77**
147	U U G U G U	41.94
148	U C G U A C	44.67
149	U U U U C G	46.27
150	U U U U A U	46.34
151	**U G A U G C**	**46.40**
152	**U C U U A G**	**46.67**
153	**U C G U A G**	**48.25**
154	U U U U U A	51.32
155	U C A U G U	53.94
156	U A U U G C	56.34
157	U C C A A A	60.99
158	**U A G U G C**	**62.91**

37.92 ± 3.38

[a]All mutants were cloned in the pOFX302 plasmid. The frequency of frameshifting was determined by pulse labeling. Construct 17, corresponding to the wild-type sequence, and constructs with stop codons are indicated in bold. Each of the four boxes contains mutants in which SP2 starts with the same nucleotide. Within a box, the mutants are ranked by increasing frameshifting efficiency. The average value and its 95% confidence interval are indicated below each box.

TABLE 2. All variants of the first two positions of spacer 2.[a]

spacer 2	%fs	spacer 2	%fs	spacer 2	%fs	spacer 2	%fs
GGUACC	10.83 ± 3.78	AGUACC	11.99 ± 1.89	CGUACC	19.35 ± 2.08	UGUACC	21.52 ± 3.87
GAUACC	14.34 ± 3.25	AAUACC	15.71 ± 3.91	CAUACC	24.76 ± 6.75	UAUACC	25.00 ± 10.3
GCUACC	**11.87 ± 3.61**	ACUACC	18.20 ± 4.16	CCUACC	30.17 ± 7.40	UCUACC	29.95 ± 7.51
GUUACC	6.04 ± 2.24	AUUACC	11.54 ± 4.89	CUUACC	16.18 ± 1.78	UUUACC	24.95 ± 5.13
GNUACC	10.77 ± 1.91	ANUACC	14.36 ± 1.87	CNUACC	22.61 ± 3.32	UNUACC	25.36 ± 2.55

[a]Each value, reflecting frameshifting efficiency, is the mean (±95% confidence interval) of four independent measurements by pulse labeling. Each box contains four mutants in which SP2 starts with the same nucleotide (wild-type SP2 is in bold); the last line is the calculated average value and its 95% confidence interval.

change at position 4 of SP2 can dramatically affect frameshifting.

Stop and rare codons

Rare codons, which are read by low abundance tRNAs, and stop codons adjacent to shift sites constitute a special issue to which some of our mutants are directly relevant. After entering the A-site of the ribosome, both types of codons are decoded more slowly than the others, thus giving more time for a recoding event to occur if a shifty motif is in the P-site and overlap either with the A-site (for +1 frameshifting) or with the E-site (for −1 frameshifting). Thus, placing a stop (or rare) codon next to a shift site is a way to test in which configuration the recoding event takes place. If the stop codon has a marked effect when compared to regular sense codons, then frameshifting is deduced to involve re-pairing of the P-site tRNA (and possibly the E-site tRNA also). If there is no effect, then frameshifting is likely to proceed via re-pairing of the A-site tRNA with, in the case of X XXY YYZ heptameric motifs, the simultaneous re-pairing of P-site tRNA (Jacks et al., 1988; Weiss et al., 1989).

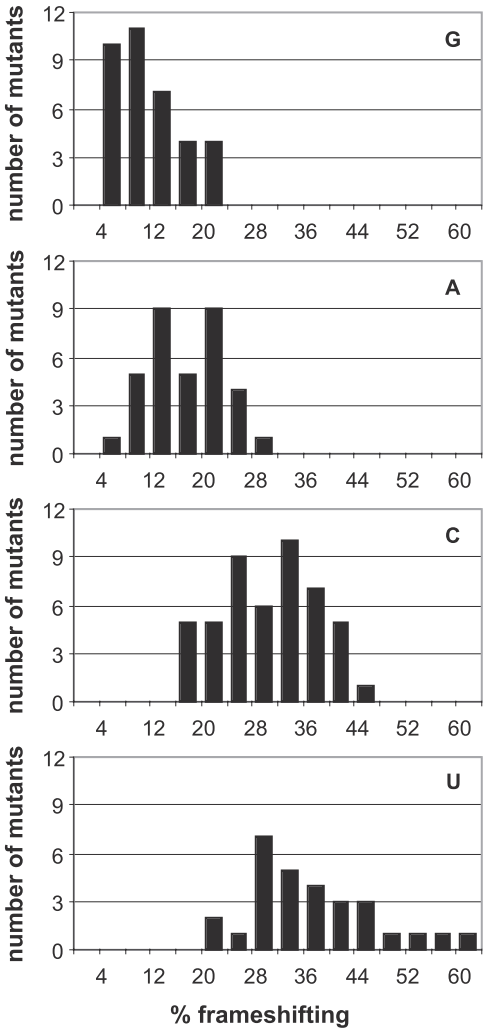


FIGURE 2. Distribution of the spacer 2 mutants from Table 1 as a function of the frequency of frameshifting.

Among our mutants, eight possess a stop codon in the 0 phase 3′ adjacent to the shift site (mutants 122, 132, 137, 142, 145, 146, 151, and 158 in Table 1) and six have a rare codon at the same position (AGA for 37 and 45, AGG for 39 and 49, AUA for 51 and CUA for 81). The results with none of them stand out: they all have a level of frameshifting within the range of the other mutants starting with the same nucleotide. Thus, in these conditions, a stop or rare codon next to the A AAA AAG heptameric motif does not increase or decrease frameshifting in any notable way.

Influence of reporters and stimulators

The 3′ context effect was demonstrated for SP2 mutants cloned in the pOFX302 plasmid. It could therefore have been due to some unique properties of this reporter system. To test this possibility, three other reporter systems were used to clone the wild-type IS*911* region and the three variations of the first nucleotide of SP2. In the first, the IS*911* frameshift region was cloned between the *gst* and *malE* genes. In the second, this region was inserted at the end of the *dhfr* gene. In the third, most of the *lacZ* gene of pOFX302 was deleted. The level of frameshifting was measured by the pulse-labeling assay for all these plasmids (Fig. 3A). Even though the level of frameshifting varies with the re-

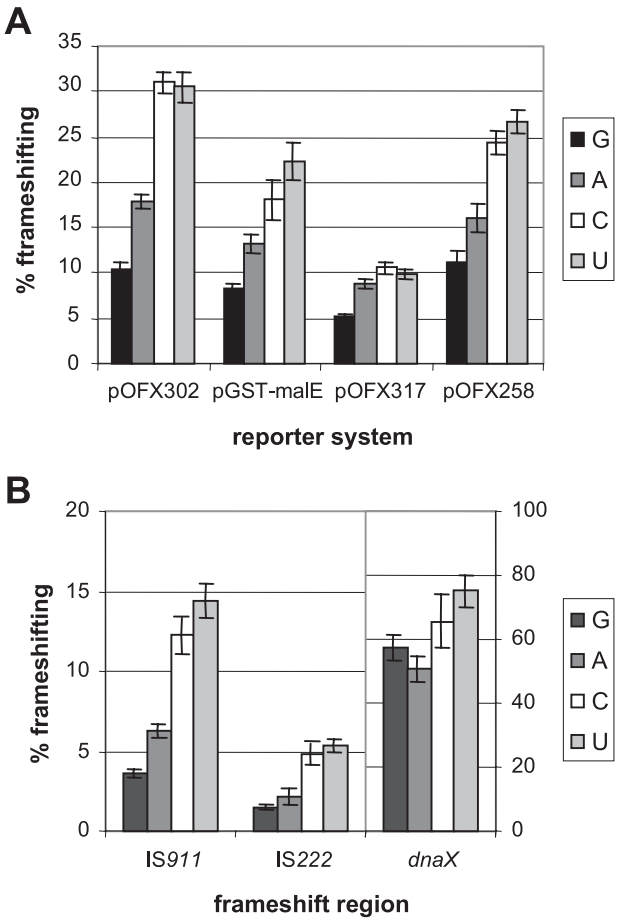


FIGURE 3. Incidence of reporters and stimulators. **A**: The wild-type region (Fig. 1) and the three mutants, in which the first nucleotide of SP2 is changed to A, C, or U, were cloned in the pGM-1, pOFX258, and pOFX317 vectors. Frameshifting frequency was measured by pulse-labeling on four clones for each construct (the 95% confidence interval of the mean is indicated). **B**: The frameshift regions of IS222 (Kropinski et al., 1994) and dnaX (Larsen et al., 1997) were cloned into pOFX302. Frameshifting frequencies were estimated by β-galactosidase assay; note that for the IS*911* constructs this method gives values three times lower than pulse labeling.

porter system, a similar 3′ context effect is observed for all of them. Thus, the 3′ context effect is not dependent upon the larger context in which the IS*911* frameshift region is placed. It is an intrinsic property of this region.

This raised the question of whether the effect of the 3′ nucleotides was specific for the IS*911* shift cassette or was also operative with other −1 frameshift signals. To answer that question, we used the *dnaX* (Larsen et al., 1994) and IS*222* (Kropinski et al., 1994) frameshift regions. These two regions of programmed −1 frameshifting are similar to that of IS*911.* They use the same heptameric motif, A AAA AAG, and both have an upstream Shine–Dalgarno sequence. A stimulatory stem-loop is present 6 nt 3′ of the *dnaX* shift site (Larsen et al., 1997) but not in IS*222*. In spite of these similarities, the nucleotide sequence of these regions is different, as are their respective frameshifting efficiencies. By pulse-labeling measurement (data not shown), the IS*222* region is two times less active and the *dnaX* region is five times more efficient than the IS*911* region. We carried out the mutagenesis of the 3′ context in these two other −1 frameshift regions cloned in pOFX302 and measured the frequency of rephasing of each of the eight resulting constructs by β-galactosidase assay (Fig. 3B); this assay gives lower frameshift frequencies than the pulse-labeling technique without affecting the hierarchy between constructs. With the IS*222* frameshift cassette, modifications of its 3′ context induce a variation in frameshift efficiency similar to the IS*911* pattern. For the *dnaX* cassette, modifications of the 3′ context cause a smaller effect on frameshifting efficiency (maximum factor of 1.5), but nevertheless the A and G mutants are significantly less efficient than C and U mutants. Thus the 3′ context effect appears as a general property of A AAA AAG-based prokaryotic −1 frameshift sites.

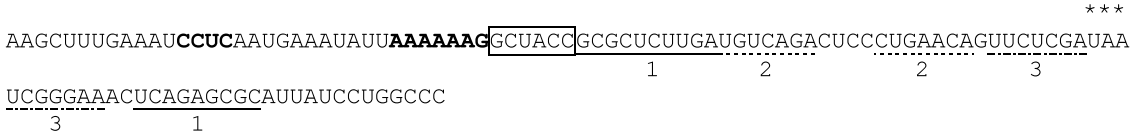
The previous set of experiments showed that the general or local environment of the A AAA AAG motif did not modify qualitatively the 3′ context effect. Because in all the situations tested stimulators of frameshifting were still present, we could not exclude that the 3′ context effect was due to the coordinated action of the first nucleotide of SP2 and of the stimulator elements. So the third environmental determinant we analyzed was the influence of the Shine–Dalgarno sequence and the stem-loop structure. The stimulator sequences of the IS*911* region were mutated in a set of six spacer 2 mutants, generating for each a deletion of the stem-loop region, a mutation of the Shine–Dalgarno, or the combined changes (Fig. 4). The β-galactosidase activity of all the mutants was measured. Deletion of the stem-loop structure causes a drop in frameshifting efficiency by a factor of about twofold, mutation of the Shine–Dalgarno sequence causes a greater drop in frameshift (about 3- to 10-fold), and combining the two mutations further decreases frameshifting (about 10- to 20-fold). The important outcome is that the 3′ context effect persists in all cases: The constructs with G or A as the first nucleotide of SP2 still display a lower level of activity than those with C or U. The conclusion is that the 3′ context effect is qualitatively independent of the stimulators.

Different heptanucleotides

The A AAA AAG motif, found in *dnaX* and in many insertion sequences of the IS*3* family, is probably the most efficient heptameric −1 shift motif in *E. coli*. But other heptamers are used for −1 programmed frameshifting, such as A AAA AAC in IS*1* (Sekine & Ohtsubo, 1992), U UUA AAG in *Salmonella typhimurium* phage MB78 (Kolla et al., 2000), and A AAA AAA in IS*600* (M.F. Prère & O. Fayet, unpubl.). To determine if the 3′

IS*911* ΔSL mutant sequence

IS*911* SD_B^0 mutant sequence



spacer 2	WT	ΔSL	SD_B^0	ΔSL SD_B^0
GCGAUC	248 ± 12,5	55 ± 3,20	47 ± 3,60	12 ± 0,90
GCUACC	121 ± 5,64	44 ± 5,33	41 ± 1,13	13 ± 0,82
AGACGC	101 ± 5,32	57 ± 5,77	43 ± 4,00	16 ± 1,29
CCGCGA	845 ± 38,5	479 ± 32,1	87 ± 4,27	69 ± 4,20
UCGAGA	701 ± 34,2	402 ± 27,8	89 ± 3,68	43 ± 2,91
UUCGAC	397 ± 44,4	323 ± 11,7	64 ± 4,31	39 ± 1,43

FIGURE 4. The 3′ context effect does not require the frameshifting stimulators. Wild-type SP2 and five variants were cloned in three modified IS*911* frameshift regions: In the first (SD_B^0 mutant), the upstream SD (in bold) has been changed from GGAG to CCUC. In the second (ΔSL), most of the downstream stem-loop structure has been deleted (the three stems are underlined and the AUU initiation codon has been mutated to ACU). In the third (SD_B^0 ΔSL) both changes were combined. Frameshifting frequency was deduced by β-galactosidase assays; the results are in β-galactosidase units (eight determinations per construct) and the 95% confidence interval of the mean is indicated.

context effect was a general property of heptameric X XXY YYZ motifs, we created mutants of the IS*911* region in which 17 other motifs were combined with each of the four possible nucleotides at the first position of SP2. In Table 3, the β-galactosidase activity of these 17 motifs, plus A AAA AAG, is presented according to their 3′ nucleotide. The 18 heptamers tested can be separated into two classes according to the 3′ context effect. There is one special case, construct A AAA AAA **A**, not discussed further, because its high level of activity is due to transcriptional slippage (data not shown) rather than frameshifting.

Motifs of the first class, the four X XXA AAG heptamers, display a 3′ context effect identical, in pattern and magnitude, with that initially found with A AAA AAG. They give a relatively high level of frameshifting (from 98 to 425 β-galactosidase units), except U UUA AAG (for reasons yet unclear, U UUA restricts frameshifting); the four of them are not efficient at all in eukaryotes (Brierley et al., 1992). The common element of these motifs, the A AAG tetramer, probably fixes the pattern of the 3′ context effect.

The second class regroups all the 14 remaining motifs that do not follow the pattern of the X XXA AAG set. They exhibit in general small or nonsignificant differences in frameshifting efficiency when the 3′ context is modified. All are poorly efficient frameshifters in *E. coli* (from 0.5 to 25 β-galactosidase units), in sharp contrast with their behavior when tested in a eukaryotic system for 9 of them (A AAA AAA, A AAA AAC, A AAA AAU, C CCA AAA, G GGA AAA, U UUA AAA, C CCU UUA, C CCU UUC, and C CCU UUU; Brierley et al., 1992).

No contribution of mRNA or protein stability and tRNA's relative abundance

We then asked if the 3′ context effect was indeed due to a modulation of the level of frameshifting or to a difference in the stability of the mRNA or protein between the different mutants. To answer that question, the stability of the mRNAs and proteins was measured as described in Materials and Methods for the four constructs, in which only the first postmotif nucleotide is modified. These two sets of experiments suggest that mutations in the SP2 region do not affect notably the stability of the g10-lacZ mRNA or of the fusion protein (data not shown). These results were confirmed by the analysis of in-phase derivatives of the same four mutants: They all synthesize the same amount of fusion protein (data not shown). Therefore, the observed variation in frameshifting level results most probably from a translational effect.

The nucleotide 3′ of a heptameric shift site determines the codon that is read, either in the 0 phase or in the −1 phase, immediately after decoding of the shift site. Assuming, even if unlikely, that after frameshifting and translocation the A-site transiently accommodates 4 bases and can accept either a 0 or −1 phase tRNA, a competition between these two tRNAs could result in the observed 3′ context effect. A testable prediction is

TABLE 3. 3′ nucleotide effect on different XXXYYYZ heptamers.

	3′ context			
Motif[a]	G	A	C	U
AAAAAAG	**121 ± 11.3**	200 ± 10.8	359 ± 40.8	425 ± 42.7
AAAAAAA	17.4 ± 1.28	164 ± 17.1[b]	7.75 ± 0.56	13.2 ± 2.16
AAAAAAC	4.13 ± 0.90	4.72 ± 0.32	3.63 ± 0.69	6.24 ± 0.52
AAAAAAU	2.51 ± 0.24	3.41 ± 0.37	2.68 ± 0.29	4.10 ± 0.28
CCCAAAG	132 ± 14.0	98.5 ± 8.77	265 ± 18.6	311 ± 17.3
CCCAAAA	8.65 ± 0.70	10.1 ± 1.07	11.1 ± 1.59	14.5 ± 1.49
GGGAAAG	113 ± 6.47	136 ± 9.20	235 ± 32.3	245 ± 34.0
GGGAAAA	9.62 ± 1.33	9.01 ± 1.41	8.62 ± 1.09	14.0 ± 1.34
UUUAAAG	5.15 ± 0.73	7.80 ± 1.39	12.9 ± 1.95	24.6 ± 2.12
UUUAAAA	1.35 ± 0.75	2.15 ± 0.44	0.50 ± 0.43	6.31 ± 1.06
AAAGGGG	22.7 ± 1.21	12.2 ± 1.98	18.1 ± 0.93	22.8 ± 2.44
AAAGGGA	14.1 ± 0.72	7.21 ± 0.56	9.87 ± 1.21	17.8 ± 1.41
AAAGGGC	11.0 ± 0.97	8.18 ± 0.60	9.43 ± 1.15	12.4 ± 0.98
AAAGGGU	11.5 ± 1.56	10.9 ± 1.61	11.8 ± 1.30	15.4 ± 1.74
CCCUUUG	6.70 ± 0.45	6.15 ± 1.59	4.21 ± 0.70	7.77 ± 1.38
CCCUUUA	7.58 ± 0.68	5.61 ± 0.56	4.00 ± 0.50	5.96 ± 0.35
CCCUUUC	18.8 ± 0.92	9.64 ± 0.89	8.14 ± 1.18	14.4 ± 0.98
CCCUUUU	26.1 ± 1.40	21.6 ± 1.12	15.5 ± 1.63	23.0 ± 2.74

[a]The AAAAAAG motif, in the standard IS*911* frameshift region (Fig. 1), was changed to 17 other potentially shifty heptamers and the four variations of the following nucleotide were generated at the same time. In all these constructs, the AUU initiation codon was changed to ACU to allow measurement of frameshifting by β-galactosidase assay. The results are expressed in β-galactosidase units (eight determinations per construct) with the 95% confidence interval of the mean. The value for the wild-type sequence is in bold.

[b]The high level of activity of this construct results from transcriptional slippage and not from translational frameshifting (data not shown).

that changing the balance between the 2 tRNAs should modify the ratio of the shifted to nonshifted products. To achieve that, we cloned the genes encoding tRNAs corresponding to two situations. In the wild-type IS*911* frameshift region (A AAA AAG GCU), the first postmotif codon in the 0 phase is GCU and in the −1 phase it is GGC. In the modified IS*911* region A AAA AAG CCU (threefold induction of frameshifting, data not shown), the first postmotif codon in the 0 phase is CCU and in the −1 phase it is GCC. The same tRNA (Ala_2) decodes GCU and GCC, GGC is decoded by tRNA-Gly_3, and CCU is read by tRNA-Pro_2. The genes of these three tRNAs were cloned behind a tetracycline-inducible promoter in a plasmid compatible with the pOFX302 reporter. The production of fusion protein was monitored by pulse labeling after induction by a factor of three of the expression of the 0 or −1 phase tRNA genes. No variation in the amount of frameshift product was seen in these conditions (data not shown). This result is corroborated by a reexamination of the data in Table 2, taking into account for each mutant the known abundance of the tRNAs reading the first postmotif codon in the 0 or −1 phase (Dong et al., 1996). Therefore, variations in the respective amounts of the first postmotif tRNAs, those decoding the 0 or the −1 phase, are probably not responsible for the 3′ context effect. However, because the translation rate of a codon is not always correlated with tRNA abundance (Bonekamp et al., 1989), it remains remotely possible that a competition between the 0 and −1 phase tRNAs occurs at a qualitative level.

DISCUSSION

The immediate 3′ context of a heptameric shift site has been shown to have a surprisingly large effect on −1 frameshifting in *E. coli*. Changing the 3′ context of the A AAA AAG motif of the IS*911* recoding region can turn a moderately efficient programmed frameshift signal (about 10% frameshifting) into a highly efficient one (e.g., up to 60%), as good as the *dnaX* signal (Larsen et al., 1994). Of the 6 nt 3′ to the shift site constituting the SP2 spacer, the identity of the first nucleotide is the primary determinant of the context effect. The order leading to diminishing frameshifting is U > C > A > G. However, the effect of the following 5 nt in SP2 can supersede the dominant role of the first nucleotide. Two independent and additive effects are probably operating: (1) a clear effect of the first postmotif nucleotide by itself, discussed in more detail below, and (2) the effect of five others, or of the six, as a whole. Recent structural studies by Noller and coworkers (Yusupova et al., 2001) suggest that the SP2 segment of the mRNA must be enclosed in a narrow channel within the ribosome when the AAA and AAG codons of the heptamer are in the P- and A-sites, respectively. SP2 therefore cannot be engaged in any pairing with a downstream sequence at that time. As suggested by Kim et al. (2001),

when the stem-loop abuts the ribosome, the SP2 spacer segment would have to adopt an extended conformation in order to get the slippery motif in the P- and A-sites, which would result in a tension on the mRNA. The tension could be released either by pulling the message back slightly, with an ensuing change in reading frame, or by melting of the secondary structure. Conceivably, the propensity to adopt this extended conformation could be influenced by the sequence in itself, and perhaps by its interaction with ribosomal elements in the mRNA channel. Importantly, the observed context effect is not an idiosyncratic property of the IS*911* frameshift region, but is a general feature of X XXA AAG shifty heptamers, independent of the presence or absence of any of the two known stimulators of −1 frameshifting (upstream SD and downstream stem-loop).

Several studies have revealed or suggested that the immediate 3′ context of a sense or stop codon can influence its translation at various steps and through different mechanisms (reviewed by Buckingham, 1994). In the following, we will discuss those most relevant to our work on −1 frameshifting. By immediate 3′ context, we mean the 1 to 9 nt following a given codon. This definition excludes the more distal and lengthy 3′ "classical" recoding stimulators, whether stem-loops or pseudoknots. Two categories of immediate 3′ context effect can be distinguished. The first can be designated in short as "P-site–A-site interaction" and covers interactions between the P- and A-site codons and/or tRNAs, ribosomal elements, and other protein factors, such as release factors, involved in translation. In that category falls the effect of stop codons on recoding on the U UUU UUA sequence (Horsfield et al., 1995): −1 frameshifting was found to be diminished by a downstream adjacent stop codon in the 0 phase. Entry of a stop codon in the A-site somehow prevents mRNA slippage and therefore affects reading at the P-site, suggesting that frameshifting occurs while the UUU and UUA-decoding tRNAs are in the E- and P-sites (Horsfield et al., 1995). In contrast, in our equivalent mutants, an adjacent stop codon does not have an effect on frameshifting (Table 1). This result is in agreement with those Jacks et al. (1988) and Brierley et al. (1997) obtained in eukaryotic systems with other shifty heptamers. It provides further evidence that the classical model of simultaneous re-pairing of the P- and A-site tRNAs also applies to recoding in *E. coli* on the A AAA AAG motif (Weiss et al., 1989; Tsuchihashi & Brown, 1992). The discrepancy with Horsfield et al. (1995) nevertheless raises an interesting possibility: In *E. coli*, at least, different motifs could be affected differently by a nearby stop codon because they do not promote frameshifting at the same time during the elongation cycle.

The second category of immediate 3′ context effect can be defined in short as a "codon-3′ sequence interaction." It concerns interactions of an A-site codon and/or tRNA with the 3′ sequence of the mRNA. For example, in *E. coli*, the suppression of UAG amber codons was found to be highly sensitive to the 3′ immediate context (Bossi, 1983; Miller & Albertini, 1983; Pedersen & Curran, 1991). The suppressor tRNA involved, an allele of *glnV* (*supE*), is a glutamine tRNA in which the 3′-GUC-5′ anticodon has mutated to 3′-AUC-5′. The observed range of variation was about 35-fold. The first rule deduced is that a purine directly 3′ to the UAG codon allows better suppression (4.5-fold) than a pyrimidine. An additional rule (Stormo et al., 1986) is that a U in the second position after the stop codon is also stimulatory (threefold). Both effects are independent and additive and can be further modulated in an unknown way by the other nucleotides in the vicinity (twofold). A likely hypothesis to explain the 3′ context effect on UAG suppression is stabilization of the codon–anticodon complex by stacking of the following base of the message on the mRNA-suppressor tRNA helix (Ayer & Yarus, 1986; Stormo et al., 1986; Pedersen & Curran, 1991). As shown in vitro by Freier et al. (1986), a purine dangling 3′ of a G-ending RNA duplex has a higher free-energy contribution than a pyrimidine. Thus, when UAG is read as a sense codon by the glutamine suppressor tRNA, having a purine as the next base on the message improves the efficiency of decoding.

The effect of the immediate 3′ context on −1 frameshifting on X XXA AAG motifs also belongs to the "codon-3′ sequence interaction" category. Like the variation in the efficiency of UAG suppression, its primary component can be interpreted in terms of modulation of the stability of the codon–anticodon complex via a stacking interaction with the next base. When the X XXA AAG motif is read, entry of the second 0 phase codon, AAG, into the A-site leads either to the normal reading frame being maintained or, after dissociation of the AAG:$tRNA^{Lys}$ complex, slippage backwards and re-pairing of $tRNA^{Lys}$ with the mRNA in the −1 frame at the AAA codon. Any factor increasing the stability of the initial codon–anticodon interaction, for example, the stacking of an adjacent purine, should favor frame maintenance and therefore limit frameshifting. Conversely, any element decreasing the stability of the complex, for example, a poor stacker like an adjacent pyrimidine, should make frameshifting easier. The additional information provided by the −1 frameshifting assay is a differentiation between the 4 bases: if the above hypothesis is correct, of the purines, G would be a better stabilizer than A, and of the pyrimidines, C would be better than U; no significant effect of the second base after the motif can be seen in our mutants.

If all the data concerning the contribution to duplex stability of 3′ dangling bases are taken into account (Table 3 in Freier et al., 1986), a prediction is that the purine versus pyrimidine effect should exist whatever the last base of the codon is. Therefore the same context effect should exist in any organism for other frameshift sites. In eukaryotes, the context effect for shifty

heptamers has not been analyzed so far. In the case of *E. coli*, our data, although partial (18 motifs analyzed out of 64), do not entirely support the above prediction. Only the 4 motifs ending with A AAG display in a clear cut manner the predicted context sensitivity; 3 of them are also the most shift prone. In our opinion, the emergence of context sensitivity (and of high shiftiness also) has a prerequisite, a weak initial pairing of the tRNA. As discussed below, it is also true for the context effect on UAG suppression. In *E. coli*, only one tRNA decodes the AAA and AAG lysine codons. It is well known that the modified U at position 34 of $tRNA^{Lys}$ pairs substantially better with third codon base A than G (reviewed in Yokoyama & Nishimura, 1995). The reason for that is not entirely clear. Agris et al. (1997) suggested that it could be due to an unconventional structure of the $tRNA^{Lys}$ anticodon. However, this proposal is not supported by recent structural analyses (Benas et al., 2000; Sundaram et al., 2000). When the AAG codon of an X XXA AAG motif enters the A site of the ribosome, the weak initial pairing of the third codon base helps dissociation of the codon:anticodon and, after mRNA slippage, stronger re-pairing of the tRNA with mRNA at the overlapping AAA codon locks translation in the −1 frame (Tsuchihashi & Brown, 1992). The nucleotide 3′ of the AAG codon possibly modulates the frequency of dissociation of the 0 frame codon:anticodon complex by stacking over the "weak" U34:G pair. If the 0 frame codon is AAA (e.g., G GGA AAA motif), then the initial pairing is good and whatever the next nucleotide is, its contribution to the stability of the codon:anticodon is too low to have a major impact on frame maintenance. Good or satisfactory initial pairing could therefore be the reason for the nonexistent or moderate context effect seen with the other heptamers tested.

In the case of UAG suppression, weakness of the interaction with the mutant tRNA cannot have its source in the perfectly matching anticodon (3′-AUC-5′). A likely candidate is nt 37, whose modification is known to contribute to codon:anticodon stability (reviewed in Yokoyama & Nishimura, 1995). The original *glnV* product has a small-sized modification of A37, m^2A (2-methyl-A). Mutation of base C36 of its anticodon to A made it capable of reading UAG stop codons as glutamine. However, in *E. coli*, nearly all tRNAs with an A at position 36 have a bulky modification on A37, ms2i6A (2-methylthio-N^6-isopentenyl-A), whose enhanced stacking contribution compensates for the relative weakness of the A36:U pair. The suppressor tRNA not possessing this stabilizing modification (Inokuchi et al., 1979) probably forms a relatively unstable codon:anticodon complex that is therefore sensitive to the identity of the base 3′ to the UAG codon. Similarly, weakening codon:anticodon interaction through hypo modification of base 37 can affect −1 frameshifting. Changing nucleotide 37 of mammalian $tRNA^{Phe}$ from the bulky imG (wyosine or Y base) to the smaller Gm (2′-*O*-methylguanosine) makes it more frameshift prone on U UUU UUU when tested in rabbit reticulocyte lysate (Carlson et al., 2001).

In the case of −1 frameshifting on A AAA AAG, we showed that the context effect operates whether recoding stimulators are present or not. Is this finding from constructed sequences reflected at potential sites of programmed and nonprogrammed −1 frameshifting especially where strong recoding elements do not dwarf the effect as in the *dnaX* region? To answer this question, we examined the 3′ context of X XXA AAG motifs in *E. coli* genes and compared it with the 3′ context of AAG codons not included in a shifty heptamer. In this latter category, out of 13,933 codons, 49% are followed by a purine (23% for G and 26% for A). Among the 144 potential shift sites, most of which do not have flanking stimulators of the known types and are therefore probably not programmed frameshift sites, the proportion is of 60.4% in favor of purines (36.8% for G and 23.6% for A). If this difference is not random, as suggested by statistical evaluation, it implies that in *E. coli*, the association of a shift-prone motif and a stimulatory 3′ pyrimidine tends to be avoided, perhaps to limit possible negative effects caused by expression of unwanted products through −1 frameshifting at nonprogrammed sites.

MATERIALS AND METHODS

Bacterial strain and growth conditions

The *E. coli* K12 strain JS238 (MC1061, *araD* Δ*(ara leu) galU galK hsdS rpsL* Δ*(lacIOPZYA)X74 malP::lacI^Q srlC::*Tn*10 recA1*) was used for all experiments.

Bacteria were grown in LB medium (Sambrook et al., 1989) or in MOPS medium (Neidhardt et al., 1974) supplemented with glucose (0.5%), thiamine (2 mg/L) and two alternative amino acid sources; the MOPS-AA version contains all amino acids at 50 mg/L each (except methionine, tryptophan, and tyrosine), and MOPS-CSA is supplemented with Difco casaminoacids (0.5%). Rambach agar plates (Merck) were used to identify clones expressing β-galactosidase. Ampicillin (40 mg/L) plus oxacillin (200 mg/L), and kanamycin (25 mg/L) were added when necessary.

DNA techniques and quantitation of radioactive macromolecules

Plasmid DNA was prepared using the Qiaprep or Qiagentip100 purification systems as recommended by the supplier (Qiagen). Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs. Ampli*Taq* DNA polymerase and the Amplicycle sequencing kit were from PE-Applied Biosystem. Cloning, transformation, agarose gel electrophoresis, and sequencing gels were carried out according to standard procedures (Sambrook et al., 1989). Radioactive products ([γ-^{32}P]ATP, [γ-^{33}P]ATP and [^{35}S]methionine) were obtained from Amersham. The Fuji X BAS1000 PhosphorImager and the PCBas software were used for the quantitative analysis of electrophoresis gels in which radioactively labeled molecules were separated ([^{35}S]-labeled pro-

teins, [γ-^{33}P]-labeled reverse transcription DNA fragments, and [γ-^{32}P]-labeled tRNAs northern blots).

Plasmid constructions

For the analysis of the frameshifting regions, we mostly used the reporter plasmid pOFX302 described by Rettberg et al. (1999). Plasmid pOFX317 was derived from pOFX302 by deleting most of *lacZ*. Plasmid pOFX302 was digested with *Bsu*36I (nt 238 to 244 in *lacZ*) and *Bsi*WI (nt 2786 to 2791 in *lacZ*). Two complementary oligonucleotides were ligated into the plasmid backbone, giving, on the coding strand, the sequence tgactaataagcttgagttagc, in which taa is the new stop codon in the *lacZ* phase. From the *Apa*I site (GGC codon) to the stop codon, the *lacZ* sequence is reduced to 75 nt. Plasmid pQE40 (Qiagen) was modified to generate pOFX258. The original vector contains the mouse DHFR gene under control of an IPTG inducible promoter. We inserted at the end of the DHFR gene a linker containing *Hin*dIII and *Apa*I sites. For that, pQE40 was digested with *Bgl*II and *Hin*dIII and two complementary oligonucleotides were inserted in between, bringing the following sequence on the coding strand: gatcggtaagcttgggcccagttaacatcgatcatcaccatcaccatcactaaag atc. The agc codon of the new *Hin*dIII site is in the DHFR reading phase, and the ggc codon of the *Apa*I site is in phase with the downstream taa stop codon. When the standard IS911 frameshift region (Fig. 1) is cloned in pOFX258, DHFR ends at the *orfA* strop codon present in the stem-loop structure. If there is -1 frameshifting, DHFR will be extended up to the taa stop codon after the *Apa*I site, giving a longer product. The pGM-1 plasmid (Moore et al., 2000) allows insertion of a frameshift region between the GST and *malE* genes via *Bam*HI and *Eco*RI sites. The various mutants of the IS*911* frameshift region (Fig. 1) were recloned after PCR amplification with 2 oligonucleotides (1097 gtacggatccgtgctgg taccgcggattc, and 1098 catagaattcacggccagtgaattagggcc), bringing terminal *Bam*HI and *Eco*RI sites with the proper phasing. The *dnaX* frameshift region (see Fig. 1 in Larsen et al., 1997) was cloned between the *Hin*dIII and *Apa*I sites of pOFX302; the four variations of the nucleotide following the A AAA AAG motif were generated using the appropriate oligonucleotides for the cloning. The same procedure was used to generate the four variants of the frameshift region of insertion sequence IS*222* (the cloned sequence is from nt 299 to nt 397 in the published sequence; Kropinski et al., 1994).

The tRNA genes (*alaW*, *glyW*, and *proL*) were cloned using the strategy of Normanly et al. (1986). Each gene was constructed from eight oligonucleotides containing the tRNA sequence and the *rrnC* transcription terminator flanked by *Pst*I and *Nhe*I sites. These oligonucleotides were ligated into pOFX512. This plasmid is derived from pMPMK6Ω (Mayer, 1995) by inserting a *Nhe*I-*Sph*I-*Bsi*WI-*Pst*I-*Eco*RI (gctag caatgcatgctctcgtacgttatctgcag) linker between the *Bgl*I and *Eco*RI sites. Then, the $P_{LtetO-1}$ promoter (Lutz & Bujard, 1997) was inserted, using four overlapping oligonucleotides, between the *Bst*EII and *Eco*RI restriction sites (gtaacctccctatcagtgata gagattgacatccctatcagtgatagagatactgagcacatcagcagg).

β-galactosidase assay

For each strain, eight tubes containing 0.5 mL of Mops-CSA were inoculated with independent clones and incubated overnight at 37 °C. These cultures were diluted 50-fold in 0.5 mL of the same medium and incubated for 2 h at 37 °C. The absorbance at 600 nm was measured on 125 μL of culture in a 96-flat-bottomed-wells microplate (optical path of 0.38 cm) with a spectramax 340PC spectrophotometer (Molecular Devices). We centrifuged 250 μL of culture, cells were resuspended in 250 μL Z* buffer (Z buffer from Miller, 1992, supplemented with 0.005% SDS, 1 mg/mL BSA, and 10 mM DTT instead of β-mercaptoethanol) and disrupted by sonication. In a 96-well microplate, a variable volume of this extract was completed to 100 μL with Z* buffer and 25 μL of 4 mg/mL ONPG were added. Absorbance was read at 420 nm each minute over a 30-min period with a Spectramax 340PC spectrophotometer. To be directly comparable to those obtained with the classical protocol of Miller (1992), our specific activities were calculated for a volume of extract of 125 μL and for an OD_{600nm} of 1.

In vivo pulse labeling of protein

These assays were carried out on two independent clones for the SP2 mutants, and on four clones for the other mutants, following the protocol previously described (Rettberg et al., 1999). The medium was supplemented with anhydrotetracycline at 200 ng/mL in the tRNA overexpression experiment. To calculate the frequency of frameshifting, the fraction of the total radioactivity present in the relevant band was divided by the corresponding value obtained for the in-phase control (strain containing the pOFX302-361 plasmid; Rettberg et al., 1999).

tRNA isolation and detection

The tRNA and aminoacyl-tRNA levels were measured by an adaptation of previous protocols (Varshney et al., 1991; Bourdeau et al., 1998). Cultures were incubated overnight at 37 °C in MOPS-AA, diluted 25-fold in the same medium containing 200 ng/mL anhydrotetracycline and maintained in log phase by dilution over a 3-h period. Two OD_{600nm} units were taken at different times to follow the expression of the $P_{LtetO-1}$ promoter. Cells were pelleted and chilled on ice and the tRNAs were extracted in acidic conditions (Bourdeau et al., 1998). The concentration of each preparation (20 μL) was determined by measuring the absorbance at 260nm of a 1:100 dilution, and all preparations were adjusted to the same concentration. Samples (2 μL) of each RNA preparation were distributed into two tubes: one was maintained at 4 °C, whereas the other was incubated at 37 °C for 25 min after addition of 1.5 μL of 0.5 M Tris, pH 9; the Tris treatment causes deacylation of all the tRNAs. To each sample, 1.5 μL of loading buffer (Varshney et al., 1991) was added before loading on a 6.5% polyacrylamide gel (8 M urea, 0.1 M Na acetate pH 5) and run for 20 h at 500 V at 4 °C. After electrophoresis, RNA was transferred by electroblotting onto a nylon membrane. The northern blot hybridization was realized with a DNA oligonucleotide corresponding to the anticodon region of the tRNAs (positions 16 to 52 of *alaW* and *valV* and positions 21 to 57 of *proL*) and with a DNA probe for 5S RNA (positions 29 to 61 in the *E. coli* 5S sequence) 5′-end labeled with [γ-^{32}P]ATP (Varshney et al., 1991). The variation, with time, in the amount of charged tRNA was obtained after correction of the data for the lane-to-lane variation in total RNA.

Measurement of mRNA level

Cells were grown overnight at 37 °C in MOPS-CSA, diluted 50-fold and incubated for 2 h at 37 °C. Transcription from the pTac promoter was induced for 10 min and total RNAs preparation and reverse transcription were carried out as described in the transcriptional slippage section. Two reverse transcription reactions were performed on each RNA extract, one with an oligonucleotide hybridizing in the *bla* gene of the reporter plasmid (gacacggaaatgttgaatactc, expected reverse transcription fragment of 58 nt), and the second with an oligonucleotide that hybridizes in the gene *10* (ctagattgagttaatctc catgtag, expected reverse transcription fragment of 74 nt). These oligonucleotides were 5′-end labeled with [γ-^{33}P]ATP using phage T4 polynucleotide kinase. The products of reverse transcription were separated on a 6% acrylamide sequencing gel.

Protein stability

A pulse-chase experiment was carried out on four independent clones. Overnight cultures were diluted 50-fold, cells were grown in 2 mL of MOPS-AA for 2 h at 37 °C. Transcription from the pTac promoter was induced with IPTG (2 mM final), and after 10 min, 20 μCi of [^{35}S]methionine (20 pmol) were added followed 1 min later by 20 μmol of unlabeled methionine. At different times, 200 μL of each culture were taken and treated as described in the pulse labeling section.

Context analysis of *E. coli* genes

The DNA sequence of 4,290 (real and potential) coding phases from *E. coli* K12, as defined in the annotation of sequence U00096, were obtained through the Workbench server (http://workbench.sdsc.edu/). We wrote scripts for the PERL program (version 5.2.2.0 for Windows, obtained from ActiveState, http://www.activestate.com), to identify genes with correctly phased potential −1 shift sites of the X XXA AAG type (where X is A, C, G, or T) and to score the nucleotide 3′ of each motif. Another script allows scoring of the nucleotide 3′ to any in-phase AAG codon. Comparison of the frequency of the 3′ base in the two populations was performed using the following test: (1) calculation of the difference between the two frequencies and of its standard deviation, S_{dq} [if n is the number of X XXA AAG motifs, r the number of motifs followed by a given base, N the number of AAG codons, and R the number of AAG codons followed by a given base, then $Q = (r + R)/(n + N)$ and $S_{dq}^2 = Q(1 - Q)(n^{-1} + N^{-1})$], (2) calculation of the 99% confidence interval ($2.6 * S_{dq}$). The two frequencies are significantly different (with $p < 0.01$) if their difference is greater than $2.6 * S_{dq}$. This is the case when G is the 3′ base, the difference in frequency is of 14%, and S_{dq} is equal to 3.76%, which gives a confidence interval of ±9.79%.

ACKNOWLEDGMENTS

We are very grateful to Patricia Licznar for making available her unpublished data, to Isabelle Canal for expert technical help, and to Agamemnon Carpoussis for critical reading of the manuscript. This work was supported by the Centre National de la Recherche Scientifique, the Université Paul Sabatier of Toulouse, and by a grant to O.F. from the ministère de l'Education Nationale, de la Recherche et de la Technologie (programme de recherche fondamentale en microbiologie et maladies infectieuses et parasitaires). This work was also supported by a grant from the National Institutes of Health (GM48152) to J.F.A.

Received August 3, 2001; returned for revision September 10, 2001; revised manuscript received October 9, 2001

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