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In vitro selection of RNA aptamers that bind special elongation factor SelB, a protein with multiple RNA-binding sites, reveals one major interaction domain at the carboxyl terminus

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ABSTRACT

The SelB protein of *Escherichia coli* is a special elongation factor required for the cotranslational incorporation of the uncommon amino acid selenocysteine into proteins such as formiate dehydrogenases. To do this, SelB binds simultaneously to selenocysteyl-tRNA^{Sec} and to an RNA hairpin structure in the mRNA of formiate dehydrogenases located directly 3′ of the selenocysteine opal (UGA) codon. The protein is also thought to contain binding sites allowing its interaction with ribosomal proteins and/or rRNA. SelB thus includes specific binding sites for a variety of different RNA molecules. We used an in vitro selection approach with a pool completely randomized at 40 nt to isolate new high-affinity SelB-binding RNA motifs. Our main objective was to investigate which of the various RNA-binding domains in SelB would turn out to be prime targets for aptamer interaction. The resulting sequences were compared with those from a previous SELEX experiment using a degenerate pool of the wild-type formiate dehydrogenase H (*fdhF*) hairpin sequence (Klug SJ et al., 1997, *Proc Natl Acad Sci USA 94*:6676–6681). In four selection cycles an enriched pool of tight SelB-binding aptamers was obtained; sequencing revealed that all aptamers were different in their primary sequence and most bore no recognizable consensus to known RNA motifs. Domain mapping for SelB-binding aptamers showed that despite the different RNA-binding sites in the protein, the vast majority of aptamers bound to the ultimate C-terminus of SelB, the domain responsible for mRNA hairpin binding.

Keywords: aptamers; elongation factor EF-Tu; selenocysteine; SELEX; special elongation factor SelB

INTRODUCTION

Selenocysteine, an amino acid derivative of cysteine in which the thiol group is substituted by a selenol group (for a review, see Hüttenhofer & Böck, 1998a), is incorporated during translation into selenoproteins such as type I iodothyronine deiodinase (Berry et al., 1991a, 1991b), glutathion peroxidase (Chambers et al., 1986), or selenoprotein P (Hill et al., 1991). In eukaryotes, selenoproteins play important roles in development, hormone metabolism, and the immune system (Chambers et al., 1986; Berry et al., 1991a,b; Sturchler et al., 1995; Walczak et al., 1996). In *Escherichia coli* three isoenzymes of bacterial formiate dehydrogenase (H, N, and O) represent prokaryotic selenoproteins involved in anaerobic metabolism, catalyzing the oxidation of formiate to carbon dioxide (Stadtmann, 1990). In these isoenzymes selenocysteine incorporation follows translation of the selenocysteyl codon UGA, identical to the opal-codon, which is decoded by the UCA anticodon of selenocysteyl-tRNA^{Sec} (Zinoni et al., 1986; Leinfelder et al., 1988). To discriminate the selenocysteyl from an opal stop codon, the formiate dehydrogenase mRNA must contain a stable mRNA stem-loop structure located immediately 3′ of the UGA codon (Zinoni et al., 1990). Two examples of such mRNAs, formiate dehydrogenase H (*fdhF*) and formiate dehydrogenase G (*fdhG*), both contain a stem-loop structure strictly required for selenocysteine incorporation in *E. coli*. This hairpin-loop motif is recognized by a special elongation factor, SelB (Forchhammer et al., 1989), which shares extensive sequence homology with elongation factor EF-Tu in its amino terminal and central part. However, at its C-terminus, it contains an extension of 240 amino

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acids not found in EF-Tu, domain 4 (Fig. 1B). Dissection of the RNA-binding sites within SelB revealed that the N-terminal portion was able to bind selenocysteyl-tRNA^{Sec} whereas the unique C-terminal fragment exclusively bound to the hairpin structures within *fdhF* or *fdhG* mRNA (Baron et al., 1993; Hüttenhofer et al., 1996; Kromayer et al., 1996). By binding selenocysteyl-tRNA^{Sec} by its EF-Tu-like domain and also, simultaneously, the mRNA hairpin (Fig. 1) and GTP (Forchhammer et al., 1989), a quaternary complex is formed that prevents termination of protein synthesis at the stop codon and results in selenocysteine incorporation (Fig. 1A).

There is also strong indirect evidence for an interaction between SelB and the ribosome. First, because it was shown that SelB is able to hydrolyze GTP in the presence of ribosomes, it indicates that SelB interacts directly with ribosomal RNA and/or ribosomal proteins as already observed with EF-Tu (Hüttenhofer & Böck, 1998b). Moreover, ribosome-dependent GTP hydrolysis by SelB was stimulated in the presence of the *fdhF* mRNA hairpin (Fig. 1C), suggesting a conformational switch within SelB upon binding the RNA structure (Hüttenhofer & Böck, 1998b) that may also be necessary for the interaction of SelB with the ribosome. Secondly, a study by Böck et al. (1997) showed that the N-terminal portion of the SelB factor can be perfectly aligned with the canonical elongation factor EF-Tu, implicating similar modes of interaction with the ribosome. As shown for ET-Tu (Moazed et al., 1988) the α-Sarcin loop of 23S rRNA in *E. coli* seems to be the primary RNA (as opposed to protein) target for this

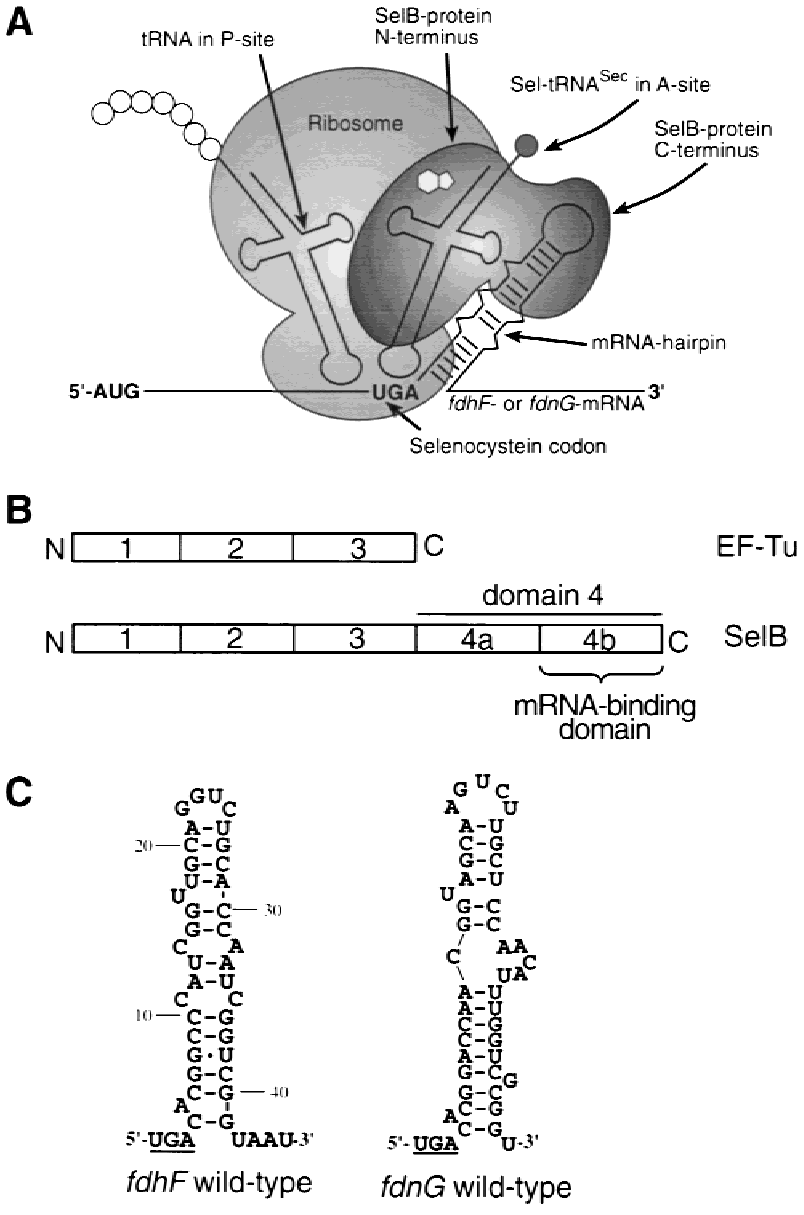

FIGURE 1. The SelB protein and the *fdhF* and *fdnG* mRNA hairpins. **A**: Schematic representation of the processes occurring during the cotranslational incorporation of selenocysteine at the ribosome. The quaternary complex between SelB, GTP (hexagon), selenocysteyl-tRNA^{Sec}, and the *fdhF* or *fdnG* mRNA hairpin are attached to the ribosome. **B**: Schematic representation of SelB and its derivatives. Bottom: the full-length SelB protein (amino acids 1–614). The protein domain 4b corresponds to the ultimate C-terminus of SelB (amino acids 472–614). In the truncated version SelB1–474 the C-terminal domain 4b was deleted. This derivative shows extensive homologies to EF-Tu (top). **C**: Secondary structures of the *fdhF* and *fdnG* mRNA hairpin motifs located immediately 3′ of the UGA selenocysteine codon.

interaction. This is supported by a recent SELEX experiment by Hornung et al. (1998), where RNA aptamers binding to elongation factor EF-Tu were isolated that contained sequence motifs resembling the α-Sarcin loop.

Taken together, these data suggest that SelB may contain three binding sites for RNA molecules: one for selenocysteyl-tRNA^{Sec}, another for the mRNA hairpin structure adjacent to the UGA selenocysteine codon, and possibly a third for ribosomal RNA. Because of the extensive sequence and structural homology between the N-terminal portion of SelB and EF-Tu, we envision that the site of ribosome interaction also resides within this domain of SelB.

Because SelB thus represents an example of an RNA-binding protein with multiple potential binding sites for different RNA molecules, we applied the SELEX-technique (for reviews, see Klug & Famulok, 1994; Gold et al., 1995; Osborne & Ellington, 1997; Jenne & Famulok, 1998) to isolate new variant RNA sequences that bind to SelB with high affinity. The main objective of our study was to investigate whether any of the three RNA-binding domains in SelB would turn out to be the prime domains for aptamer interaction, and which motifs would be selected from a completely unbiased RNA library. We also wanted to compare the resulting sequences with those from a previous SELEX experiment based on a degenerate aptamer pool derived from the wild-type *fdhF* hairpin sequence (Klug et al., 1997).

RESULTS AND DISCUSSION

In vitro selection of RNA aptamers that bind special elongation factor SelB

We performed an in vitro selection approach (for reviews, see Klug & Famulok, 1994; Gold et al., 1995; Osborne & Ellington, 1997; Jenne & Famulok, 1998) with a pool of RNA sequences totally randomized at 40 nt to select RNA aptamers that bind to the special elongation factor SelB of *E. coli*.

Purified SelB protein from *E. coli* and an RNA library of about 6×10^{14} different molecules with a length of 76 nt randomized at the central 40 nt (pool 40; Fig. 2) were subjected to in vitro selection. To avoid cross-contamination, this pool had its unique set of primer-binding sites that differed from those of a partially randomized RNA library used in this laboratory in a previous SELEX experiment (Klug et al., 1997). The SelB-binding RNA aptamers were selected by incubating SelB with the radiolabeled RNA pool and subsequent nitrocellulose filtration (Carey & Uhlenbeck, 1983; Fitzwater & Polisky, 1996). SelB-bound aptamers were extracted from the filters (Tuerk & Gold, 1990), reverse transcribed to cDNA, amplified by PCR, and transcribed in vitro back to RNA to yield an enriched pool that was used as the input for the next selection cycle. The wild-type *fdhF* mRNA sequence (wt 60.2) was included in the selection as a specific competitor for SelB binding and to ensure that a fraction of the SelB protein exists in a conformation competent for rRNA binding. This specific competitor did not contain the primer hybridization sites of the RNA library, so that it could not be reverse transcribed or amplified by PCR. Figure 3 shows the enrichment of the radiolabeled pool 40 RNA for specific SelB binding in competition with the smaller radiolabeled 60-mer wild-type *fdhF* RNA in each selection cycle performed (Bartel et al., 1991).

After one round of selection, only wild-type *fdhF* RNA binding to SelB was detected. By round two, binders from the library that compete with wild-type *fdhF*-mRNA for SelB binding began to appear. These molecules dominated the population after round three. After four rounds of selection with pool 40, SelB-binding RNA aptamers were isolated that effectively outcompeted

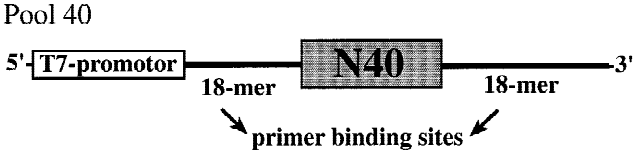

FIGURE 2. Design of the random 76-mer oligonucleotide used in the pool 40 selection. N40 indicates a complete randomization over 40-nt positions. For primer sequences and restriction sites see Materials and Methods. Pool complexities were determined as described previously (Geiger et al., 1996).

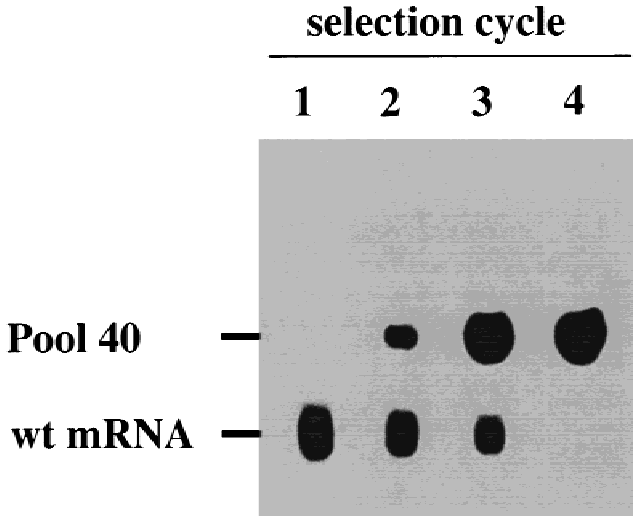

FIGURE 3. Assay of selection progress monitored by pool binding activity. 5′-^{32}P-labeled pool RNA (0.2 μM) from 1, 2, 3, or 4 cycles of selection and 0.04 μM of the shorter 5′-^{32}P-labeled wild-type *fdhF* mRNA hairpin (total length of 60 nt) were allowed to compete for binding to 10 nM SelB. After incubation for 1 h at 37 ° C one fifth of the binding reaction was removed and the rest was filtered over nitrocellulose membranes. RNA coretained with SelB on the filter was eluted, precipitated, redissolved, and subjected to electrophoresis on an 8% denaturing polyacrylamide gel.

wild-type *fdhF* mRNA for SelB binding (Fig. 3). The RNA from the final cycle was reverse transcribed, and the resulting pool of cDNA was converted to dsDNA by PCR amplification, digested with restriction enzymes *Pst*I and *Bam*HI, cloned into a pUC 19 plasmid, and sequenced.

Characterization of selected aptamer sequences

We obtained 29 different aptamer clones with unambiguously confirmed sequences (Fig. 4). Alignment of the sequences of individual selected RNA molecules revealed no obvious conserved region in most of these RNAs.

Interestingly, with two exceptions, none of these sequences share any clear sequence similarities with the wild-type *fdhF* or *fdnG* mRNA hairpins, selenocysteyl-tRNA, or any other known tRNA sequence. One of the exceptions, clone 488, however, is noteworthy among the pool 40 aptamers as this RNA has exactly the same 5′-CAAGUCUUG-3′ sequence in the apical loop (AGUCU) and adjacent stem as the mRNA hairpin of the *E. coli fdnG* gene (Fig. 5B). The upper portion of this stem-loop structure, which promotes selenocysteine incorporation into formiate dehydrogenase N, represents the SelB-binding domain of this hairpin. Followed by a 4-bp stem, the bulged U17 appears at exactly the same distance from the apical loop as in the wild-type *fdnG* mRNA. The distance between U17 and the apical loop is a constraint previously shown to be important for SelB binding (Hüttenhofer et al. 1996; Klug et al., 1997; Liu et al., 1998). Only 2 bp within the helix deviate from the wild-type consensus: the G19–C29 pair is switched to C19–G29 and the following A18–U30 pair is switched to C18–G30. Exactly the same changes were found in a previous study to be tolerated in SelB binding by the *fdhF* mRNA hairpin (Klug et al., 1997), indicative of a similar tertiary structure of both hairpins. This clone also shows a pattern of protection in enzymatic and chemical probing experiments in the presence and absence of SelB identical to that found in the wild-type *fdnG* hairpin (Hüttenhofer et al., 1996). Clone 488 thus represents a rare example of isolating a natural target-responsive sequence from an unconstrained RNA library and demonstrates that our experimental selection conditions were generally suitable for obtaining genuine SelB-binding motifs.

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    Clone Sequence

wt (fdhF) UGACACGGCC CAUCGCUUGC AGGUCUCGAC GAAUCGGUCG GU
wt (fdnG) UGACACGGAC CAACGCUAGC AAGUCUUCGU CGAACAUUUG GUCGCGG

      488 GCCCAUAAGU UGUGCCAAGU CUUGGGCGCA AAUACAUCCC
      487 CAUAUGGUGG AAAGUCUUGC CAGGUUGUGA ACGCUUCAC
      415 GAAGUUGCGG CAUGCGCAA GUUCGCGUCG CAUAUUGUGG
      498 ACGUUACCUG AAAGUUGUGG UAAGUUAGUU CUGCCGAGAG
      472 CUCAACGUGG UCGGUAUGCA UGAAGUUUGA CAGAAACGUC
      493 AGCAGCGACC CGAAGUUUUC CCAGGACUCU UUGACU
      412 AGUGGUCGGU AAGUGUUUAC UUGCGAAGUU GGUUCCAUC
      425 CCACCUGAUG GACAUUAGUU GUGGUCUAGG GCAGUGGGAA G
      491 UUGGGGCAGC AACUGGGUGA GUCCCCAGGU UGGAGGCGGG GC
      454 GGCGUUGCGG GAGUGACGAU CUUCCCGGGA AAACGUUUGU
      445 UUUCCGGAAU AGUUGGUCCG GGGUUUUAAG CUUUGAGUUG
      402 UGCCUUCUCA GUUCCGGAAU CCUCGAGGCG UUCUUCCCUC
      443 UCAUACUGCG CUACUACUAG UGAAAGGGAA UCCUGACU
      440 GUCUUCGAGA AAAUCCAGGC UGACGAUUUG ACCGUUGCAC
      450 CAUCUGACCU GAUUUAGCGA UGGGUGUAGA AUCUUGAAGC C
      480 UGAUUACCUC UUGACGUGGG CUAGGAACAU GCUGCGAUGG
      492 CGGUCACUGC UGUAUGAGCU AGCGUCAUAG GUGCAGU
      423 AAAAGCUGUG CCGACUUAGG GAAAGGGAUC UUUGACGGCC A
      448 UUAUUGGACC GAAUGCUCUU UACCCAGGUC UCCUGAAGCC
      481 AACGGUGUCC GGUAGAUUUC GUUAUGCGUA UUCAACU
      489 GGGUACCAGC UUUAUUCGUG GUCGUGACCU GGGCGAGUGG
      426 UGUAUUGGAG GUUCACUAUU UUCCAAGGUC CUGAGCCUGU
      439 UUCCACCGAC UUACAUAUGU CUUUAGAAUC CUGAUAGCC
      466 CCGUGGUCGG UAUGCUUUGU UUCAUCUGUA UGUAUGCACA
      449 GGUUCCUAUU GUGGCUACAU CGCUCACAAG GUGGAAUCUC
      475 CGCGCAUAAU GAUCUCUCCG UUGUGUCAUG GUUCGUAAC
      441 AAGGUGUCAC UAUAAUGUAC ACUAGCUUGG GCUAGAGUCG
      455 GUAUAGCGGU AUGACGCGCU AGGUAACUGG AUUGAGUGC
      499 UGCACAAGAU AUGUACGUUU GGUUACCAGG AUGCUUGCUA UG
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FIGURE 4. Sequences of selected SelB-binding RNA aptamers. Only the inserts corresponding to the randomized regions are shown. Clone 488 is aligned to the wild-type *fdnG* mRNA hairpin. The shading reflects the Watson–Crick paired helices and the UGA selenocysteine codon in the *fdnG* mRNA hairpin. The sequence regions boxed in black represent terminal loop sequences confirmed by chemical and enzymatic probing.

The second exception was clone 487, which also contains the AGUCU loop motif found in the *fdnG* wild-type mRNA (Fig. 4). However, the Watson–Crick helix that accompanies this loop is less clearly established than in clone 488. Most importantly, the bulged U17 residue, a critical determinant in SelB binding (Hüttenhofer et al., 1996; Klug et al., 1997; Liu et al., 1998), might not arise because the stem contains a possible pairing partner for this U-residue in the opposite A. This clone thus does not resemble the natural *fdnG*-mRNA hairpin as closely as clone 488.

The other RNAs represent SelB-binding molecules that do not share any obvious consensus motifs that relate them to each other. Nor do they show obvious primary- or secondary-structure relationship to the known wild-type RNAs (Fig. 4), in that they differ significantly from the sequences obtained in a previous SELEX experiment using a degenerate pool comprised of the wild-type *fdhF* hairpin sequence (Klug et al., 1997). In the previous experiment, most of the sequences isolated could be related to the *fdhF* mRNA hairpin. Here, the vast majority of aptamers isolated represent completely novel motifs competent for SelB recognition. Some of the selected sequences, however, probably possess the ability to form a hairpin secondary structure somewhat resembling the *fdhF* or *fdnG* mRNA stem-loops according to structure prediction programs (Zuker, 1989) (not shown and Fig. 5). Their relationship to these wild-type motifs is unclear, however, because they clearly lack the motif characteristics that have previously been determined as critical for SelB binding in the wild-type mRNA hairpins (Hüttenhofer et al. 1996; Klug et al., 1997; Liu et al., 1998).

The secondary structures of pool 40 RNAs proposed on the basis of the Zuker RNA folding algorithm (Zuker, 1989) were in agreement with chemical [DMS, kethoxal, CMCT, and enzymatic (nuclease S1)] probing data (data not shown and Fig. 5).

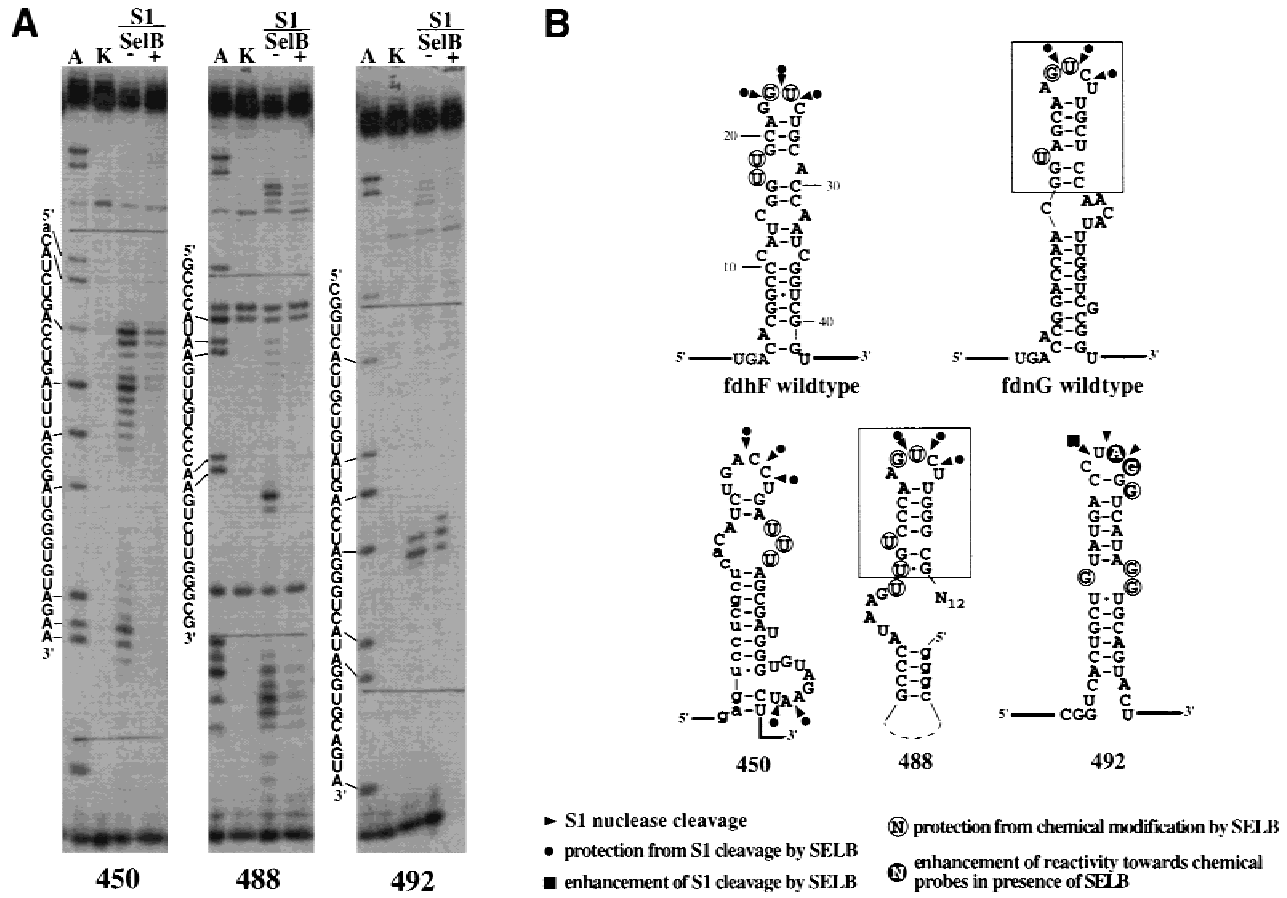

FIGURE 5. Secondary-structure determination of representative aptamer clones. Secondary structures of the *fdhF* and *fdnG* mRNA hairpins (Hüttenhofer et al., 1996) and aptamers are according to chemical and enzymatic modification analyses. **A**: Primer extension analysis of enzymatic probing with nuclease S1 in the absence or presence of SelB. Probing is shown representatively for three aptamers (450, 488, and 492). Lane A: Sequencing lane of A residues by primer extension in the presence of dideoxy adenosine triphosphate. Lane K: primer extension of untreated RNA. S1: +10 U nuclease S1. S1 + SelB: +SelB. **B**: Protection of bases from chemical modification and S1 nuclease cleavage by SelB. Positions cleaved by S1 nuclease are shown by black triangles; protection from S1 cleavage by SelB is indicated by black dots; enhancement of S1 cleavage in presence of SelB is shown by a black square. Circled bases show protection from chemical modification by SelB; enhancement of reactivity toward chemical probes in the presence of SelB is represented by white bases in a black circle. The box in aptamer 488 represents the SelB-binding domain homologous to that within the *fdnG* wild-type, which is also boxed. Bases in lower case letters are part of the constant regions.

Figure 5B shows the nuclease S1 probing pattern of three aptamer clones (450, 488, and 492) in the presence and absence of SelB. Figure 5B summarizes the chemical and enzymatic modification pattern of these clones. For clone 488, the apical loop shows the same nuclease S1 cleavage and SelB protection pattern as found previously for the *fdnG* wild-type (Hüttenhofer et al., 1996). The probing and protection pattern for kethoxal and CMCT in the apical loop and stem including the bulged U is also nearly identical to the *fdnG* wild-type sequence, confirming that this aptamer does indeed resemble the natural *fdnG* mRNA hairpin. The nuclease S1 cleavage and protection pattern for clones 450 and 492 are in accordance with the secondary structure motifs shown in Figure 5B. Clone 450 contains an apical loop and two internal bulges that are partly accessible to nuclease S1 cleavage in the absence of SelB and are inaccessible in the presence of SelB. The fact that the 5′ half of the proposed internal bulge (5′-caCA-3′) and the 5′ half of the proposed asymmetric bulge at the 3′ end (5′-UGUAG-3′) are not accessible to cleavage by nuclease S1 may indicate that these two regions might pair with each other to form a pseudo-knot motif in which the 5′-aCA-3′ at the 5′ end pairs with the 3′-UGU-5′ at the 3′ end. Additional data will be necessary to confirm this suggestion because the chemical probing experiments with CMTC, kethoxal, and DMS only allowed unambiguous conclusions for the three consecutive bulged uracil residues in the internal bulge that were protected from modification with CMCT in the presence of SelB. The secondary structure of clone 492 was clearly confirmed by both

chemical and enzymatic probing/protection experiments. In contrast to all other SelB-binding aptamers tested, however, this clone did not show protection from S1-cleavage or chemical modification of the apical loop region in the presence of SelB. In fact, S1-cleavage at one position in the apical loop was enhanced in the presence of SelB. While the internally bulged guanosine residues became protected from kethoxal modification in the presence of the protein, the guanosine and the adenosine in the apical loop showed enhanced reactivity towards kethoxal and DMS when SelB was present. This behavior might be indicative of a high degree of adaptive binding of the clone 492 aptamer to SelB.

Binding affinity of selected aptamers in competition with the wild-type *fdhF* mRNA hairpin

Some of the selected aptamers were chosen for more detailed structural and functional characterization (Fig. 6B). To compare the affinity of individual selected aptamers to SelB with that of the wild-type *fdhF* mRNA hairpin (wt 60.2), we performed a binding assay in which an excess of radiolabeled aptamer clones and the smaller labeled wild-type were allowed to compete for binding to a limited amount of SelB (Table 1; Bartel et al., 1991).

SelB/RNA complexes were allowed to equilibrate for 1 h at 37 °C and then retained on nitrocellulose filter membranes. Complexes were subsequently eluted by protein denaturation, separated according to size by denaturing polyacrylamide gel electrophoresis, and quantified by a phosphorimager.

Because of the unrestricted diversity of the RNA library, a relatively high abundance of high-affinity SelB binders unrelated to the wild-type mRNA motifs were obtained; among 12 clones for which the binding ratios were determined, six bound more than 50-fold better than the wild-type (Table 1), which exhibited a dissociation constant (K_d) of 60 nM in these experiments. This value is in agreement with a K_d of 30 nM determined previously by Hüttenhofer et al. (1996) in a chemical probing study. For comparison and to quantify the unspecific affinity of SelB to noncognate RNA molecules, we also determined the dissociation constant of the unselected pool as being 75 μM, meaning that a 1000–2000-fold increase in the binding affinity had been achieved in four rounds of stringent selection. This factor of enrichment compares well with similar in vitro selections reported for different RNA-binding proteins that led to values between 80-fold and 500-fold or higher (Shannon & Guthrie, 1991; Schneider et al., 1992; Ghetti et al., 1995). As pointed out by Irvine et al. (1991), the probability of isolating high-affinity binders in a population increases with the ratio of their individual K_d to bulk K_d.

Mapping of the SelB–aptamer interaction

The SelB protein contains at least two distinct RNA-binding domains. One is the ultimate C-terminus half of region 4b (Fig. 1B), which contains a high-affinity binding site for the two distinct naturally occurring RNA hairpins of the *fdhF*- and *fdnG*-mRNAs encoding the bacterial formiate dehydrogenases H and N (Kromayer et al., 1996). Another is the binding site for selenocysteyl-tRNA^{Sec} and is located within the N-terminal domain of SelB, a region that is highly homologous to elongation factor EF-Tu (Forchhammer et al., 1989; Böck et al., 1997), and that may also be involved in SelB interaction with ribosomal RNA and/or ribosomal proteins in a fashion similar to EF-Tu (Hüttenhofer & Böck, 1998b). The question was which region of the SelB protein had been the prime target for selecting the various and largely unrelated aptamers.

We performed a mapping experiment by testing selected aptamers for their ability to bind SelB protein derivatives in vitro (Fig. 6). The aptamers were analyzed for binding to the full-length protein, to a 17-kDa fragment (recombinant SelB comprising amino acids 472–614; Kromayer et al., 1996) corresponding to the C-terminal subdomain of SelB, and to a C-terminal truncation comprising the EF-Tu-homologous region of SelB (recombinant SelB fragment comprising amino acids 1–474; Fig. 6A; Kromayer et al., 1996) by a gel retardation assay under native conditions (Fig. 6B). For comparison, gel-shift and filter-binding assays were conducted in parallel with the wild-type *fdhF* mRNA hairpin sequence wt 60.2. Six clones chosen from pool 40 were assayed (Fig. 6B); all clones were found to bind tightly to full-length SelB protein. Among these, five interacted with the 17-kDa C-terminal SelB fragment with almost the same affinity as with the full-length SelB, binding behavior similar to that of the wild-type *fdhF*- or *fdnG*-mRNA hairpin sequences (Kromayer et al., 1996).

As expected for an in vitro selection experiment in which the only selection criterion was binding to SelB in competition with a natural SelB-binding RNA sequence, the selected pools also contained aptamers that utilize different binding sites on the protein. Such sequences are represented, for example, by clone 450 (Fig. 6C). This clone recognizes neither domain 4b nor the truncated C-terminus SelB protein but binds with high affinity to the full-length SelB protein. Interestingly, this clone is able to compete very effectively with the wild-type *fdhF* mRNA control sequence wt 60.2 for binding to the SelB protein, although the wild-type hairpin only requires domain 4b for tight interaction. Two explanations may account for this phenomenon: either the binding of clone 450 to another region of SelB interferes with the binding of wt 60.2 to domain 4b by inducing structural changes in this protein domain, or clone 450 binds to a different domain of SelB but in addition requires domain 4b for high-affinity binding.

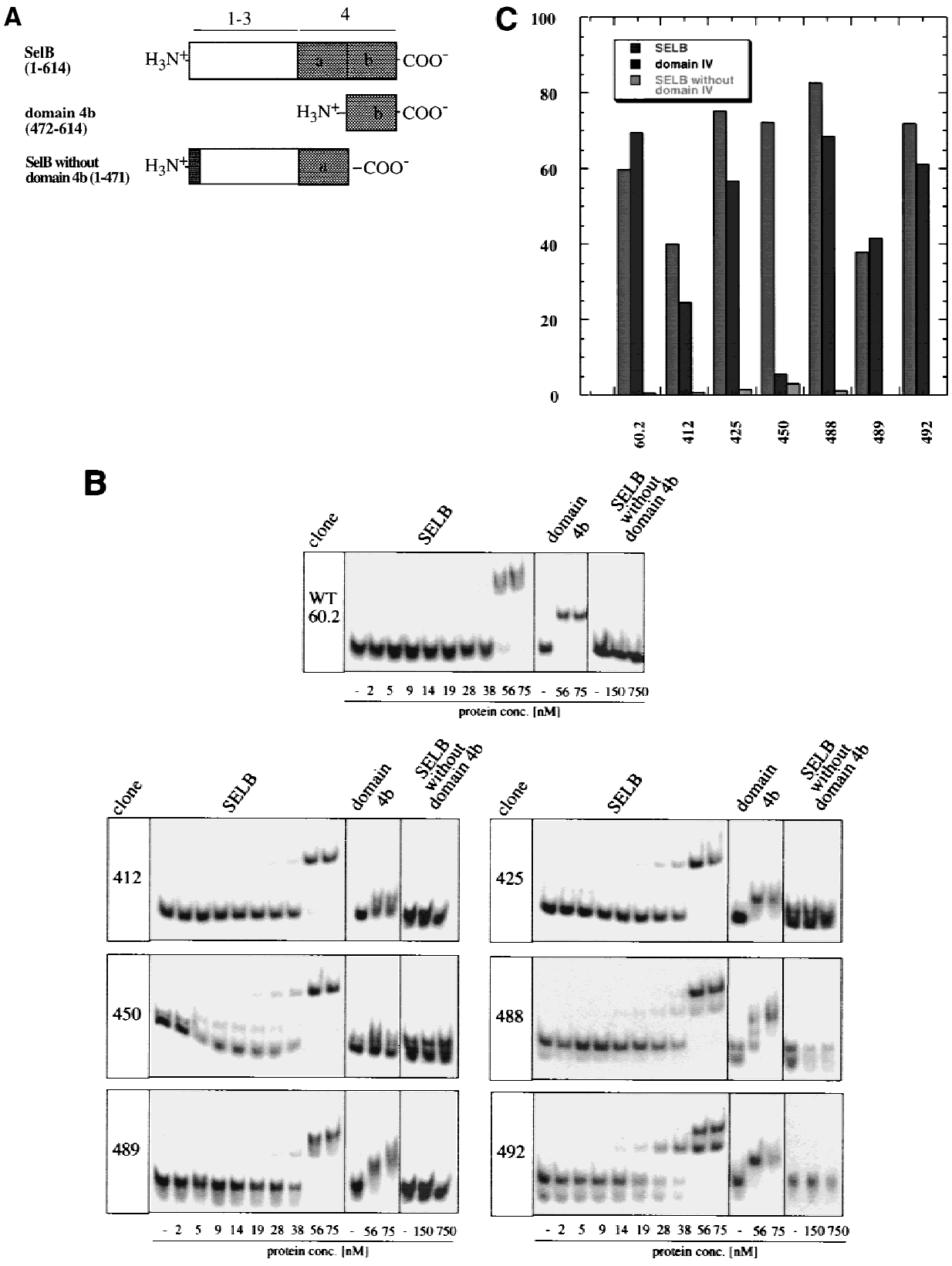

FIGURE 6. Assay of SelB binding of aptamers sampled for six aptamers. **A**: The various SelB derivatives used in this assay. **B**: Representative bandshift assays of the complex formation of six radiolabeled aptamers (75 nM) with indicated concentrations of full-length SelB protein (SelB), the C-terminal domain 4b [for the amino acid sequence of this fragment see Kromayer et al. (1996)], and the remaining N-terminal SelB fragment, homologous to EF-Tu (SelB without domain 4). **C**: Filter-binding assays of binding of the wild-type clone 60.2 and 6 aptamer clones to full-length SelB, domain 4b, and SelB without domain 4b (the EF-Tu homolog). Values show the percentage of the total radiolabeled input RNA coretained with the protein on nitrocellulose filters after extensive washing with binding buffer. RNA concentration was 15 μM; concentration of protein was 6.5 μM. The binding reaction was incubated for 1 h at 37 °C in binding buffer in the presence of 400 U/mL RNAsin.

TABLE 1. Summary of the binding activity of individual selected aptamer clones in the presence of the specific wild-type competitor RNA wt 60.2.

Pool 40 clone	Binding ratio[a]	Pool 40 clone	Binding ratio
499	>50[b]	480	>50
492	40	450	>50
491	>50	440	20
489	0.1	425	25
488	2	415	>50
481	0.5	412	>50

[a]The ability of individual selected aptamer clones to compete with the wild-type *fdhF* mRNA hairpin is reflected in the binding ratio—the ratio of radiolabeled aptamer to radiolabeled wild-type RNA of smaller size retained on the filter and subsequently visualized by PAGE.

[b]In cases marked >50, the aptamer quantitatively outcompeted the wild-type for SelB binding, so a number for the binding ratio is not given. These aptamers bind at least 50-fold better than the wild-type.

Selected RNA motifs do not resemble tRNA- or rRNA-like structures

None of the aptamers tested bound to the EF-Tu homologous N-terminal portion of the SelB protein lacking the C-terminal domain 4 (Fig. 6C) even at tenfold higher protein concentrations. In addition, we did not select any sequences that resemble $tRNA^{Sec}$ minimal motifs that might possibly bind to the N-terminus of SelB (Forchhammer et al., 1989; Kromayer et al., 1996). Although the length of the 40-mer randomized region is shorter than the average 75–79 bases of a tRNA, it should have been possible to select at least tRNA-like "mini-helices" with affinity to the $tRNA^{Sec}$-binding domain of SelB. Similar experiments were carried out in an in vitro selection by Narazenko and Uhlenbeck (1995), who isolated mini-helices of 35–44 nt binding with high affinity to EF-Tu. It should be noted, however, that our RNA constructs were not aminoacylated. Therefore, our results suggest that tRNA recognition in the N-terminus of SelB depends on the aminoacyl moiety of selenocysteyl $tRNA^{Sec}$ to a much larger extent than on sequence characteristics of the $tRNA^{Sec}$ itself. Similar results have been observed for elongation factor EF-Tu and its interaction with canonical tRNAs.

Hornung et al. (1998) conducted an in vitro selection experiment in which an RNA library completely randomized at 50 nt was selected for binding to elongation factor EF-Tu. No tRNA-like sequences or mini-helices were isolated here either. Instead, they isolated sequences that contain the consensus motif 5′-ACCG AAG-3′ also found in the α-Sarcin domain of *Thermus thermophilus* 23S rRNA. This 7-mer consensus sequence in their aptamers was found to be absolutely required for EF-Tu binding. Surprisingly, a search of our selected clones for motifs related to the ones isolated by Hornung et al. did not reveal any obvious sequence relationships to ribosomal RNA, not even to regions of the rRNAs known to interact with EF-Tu such as the α-Sarcin loop. This is additionally confirmed by the fact that almost all the aptamers found (not shown) bound to the C-terminal portion of SelB known to interact with the mRNA hairpin structure rather than with rRNA or indeed tRNA (Hüttenhofer et al., 1996; Kromayer et al., 1996).

This result has an interesting implication for what might be expected from in vitro evolution experiments in which aptamers are to be simultaneously selected for binding to proteins or protein domains with different affinities for RNA. Can one expect to select aptamers for every potential RNA-binding domain, independent from their overall affinity for RNA? One explanation for the exclusive enrichment of domain 4b binders in these experiments may be a simple statistical one, which might take effect if many more sequence solutions for binding to the C-terminal domain 4b exist than solutions for binding to other RNA-binding domains. In a situation in which such a large number of high-affinity binders for a protein or protein domain compete against a smaller number of binders for another domain or protein, the minority species might quickly be outcompeted by the many sequence solutions and become lost during the selection. Another possible explanation may be that the domain 4b binders have a higher k_{on} and/or lower k_{off} than the aptamer sequences that bind to other domains, giving these sequences a simple kinetic advantage to outcompete other binders during iterative rounds of selection and amplification.

If many more sequence solutions exist that are able to bind to the C-terminal domain of SelB, as shown in our study, why do naturally occurring SelB-binding motifs show so little sequence deviation from each other as observed with *fdnG* and *fdhF* mRNAs (for a review, see Hüttenhofer & Böck, 1998a)? One reason might be that hairpin sequences are located within the linear coding region of mRNAs, thereby putting additional constraints on the sequence and feasible conformation of the SelB-binding RNA motifs.

On the other hand, we demonstrated in a previous study (Klug et al., 1997) that mere binding of an RNA hairpin to SelB is not sufficient for incorporation of selenocysteine into proteins in vivo. One possible explanation is that this process requires that the spatial relationships of the quaternary complex comprising GTP, SelB, the mRNA hairpin, and selenocysteyl-$tRNA^{Sec}$ with respect to the ribosome have to be precisely maintained. Moreover, we recently provided evidence that SelB interaction with the *fdhF* mRNA hairpin may lead to a conformational switch within the protein that is absolutely required for its interaction with the ribosome (Hüttenhofer & Böck, 1998b). This model implies that SelB has to interact with the ribosome in a very precise way and might provide a mechanism explaining how the incorporation of selenocysteine at normal UGA stop codons is prevented. Thus, RNA motifs that bind to SelB would have to be able to induce this conforma-

tional switch and maintain the correct spatial relationships to be biologically functional. This might not be the case for all RNA aptamers isolated, even if they bind SelB more tightly than the wild-type motif.

Conclusion

After four rounds of selection for SelB binding, an RNA library randomized at 40 nt was enriched for diverse and unrelated aptamers with up to 50-fold higher SelB protein-binding affinity than the wild-type mRNA hairpin sequence. None of them bound to the N-terminal, EF-Tu homologous region of SelB, or showed any resemblance to potential SelB-binding tRNA or rRNA structures, suggesting perhaps that SelB tRNA recognition depends to a lesser extent on the tRNA sequence than the aminoacyl moiety and that ribosomal interaction may be mediated primarily by ribosomal proteins rather than rRNA.

Although almost all the isolated aptamers bound only to the SelB C-terminal domain responsible for mRNA hairpin binding, only two resembled the mRNA stem-loop structures essential for selenocysteine incorporation during translation in vivo. The fact that such a range of efficiently binding aptamers with no obvious consensus motifs or relationship to naturally occurring mRNA hairpins was obtained suggests that many more sequence solutions exist for RNA binding to the C-terminal domain of SelB than are used. This implies that the RNA motifs selected during evolution of the selenocysteine-incorporating machinery have been constrained by a number of biological functions: as a protein coding transcript, as a conformational switch required for SelB interaction with the ribosome and for maintaining the spatial arrangement of GTP, SelB, the mRNA hairpin and selenocysteyl-tRNA^{Sec} with respect to the ribosome.

The incorporation of selenocysteine into proteins has apparently evolved to be a finely tuned process requiring SelB interactions with the mRNA hairpin, tRNA, and ribosome in a very precise way. Although the selected aptamers may be biologically inactive for selenocysteine incorporation, they may prove to be highly efficient inhibitors of this process in vivo, offering alternative ways of analyzing the interactions involved.

MATERIALS AND METHODS

Pool construction

DNA pool, wild-type control sequences, and all primers were synthesized on a Millipore Expedite DNA synthesizer. The synthesized DNA included a cassette of 40 completely randomized nucleotides (in bold letters) flanked by its individual set of primer sequences (5′-GCG CTA AGT CCT CGC TCA—(**N40**)—ACGCGCGACT CggatccG-3′; 5′ primer: 5′-TCT AAT ACG ACT CAC TAT AGG GCG CTA AGT CCT CGC TCA-3′, 3′ primer: 5′-GTC gga tcc GAG TCG CGC GT-3′ including a *Bam*HI site). The synthetic single-stranded DNA was PCR amplified using an individual set of primers (5′-TCT AAT ACG ACT CAC TAT AGT CAG GAT GAC TGC TGC G-3′ and 5′-GCTTGAATCCG TAATGCTCA-3′ at the 5′ and 3′ termini, respectively; T7 promoter sequence is underlined). The wild-type competitor sequence was also synthesized and amplified in the same way using oligonucleotides 5′-CTC GTG TCT GAC ACG GCC CAT CGG TTG CAG GTC TGC ACC AAT CGG TCG GTA ATG GCG CAA-3′; 5′-primer: 5′-TCT AAT ACG ACT CAC TAT AGG CTC GTG TCT GAC ACG GCC CA-3′ and 3′-primer: 5′-TTGCGCCATTACCGACCGAT-3′. During amplification of gel-purified DNA by PCR, the T7 promoter sequence was introduced into all sequences via each individual 5′ primer.

In vitro selection

The synthetic dsDNA library was transcribed in vitro using T7 RNA polymerase. The resulting RNA pool was subjected to four rounds of in vitro selection. All cycles of selection and amplification were performed as follows: Gel-purified, α-^{32}P-labeled pool RNA was renatured by preincubation for 2 min at 70 °C, cooled to ambient temperature, and preselected for nitrocellulose binding in the presence of 400 U of RNAsin (Promega). The preselected pool was then incubated with SelB protein in a molar ratio of 20:1 (RNA:SelB) for 1 h at 37 °C in binding buffer (50 mM potassium phosphate, pH 7.0, 5 mM $Mg(OAc)_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM GTP, 0.02% Tween 20, 400 U/mL RNAsin). As a nonspecific competitor, 5S rRNA (50 μg) was used in all selection rounds and a twofold molar excess of radiolabeled wild-type 60-mer RNA hairpin was added in each round as specific competitor and to ensure correct SelB–protein conformation. Following incubation, the mixture was filtered over 0.45 μm nitrocellulose filters. Unbound RNA was removed by washing with binding buffer and RNA complexed to the protein was eluted by a procedure modified from Tuerk and Gold (1990). Glycogen (20 μg) was added to the eluted RNA solution as a precipitation support. The RNA was ethanol precipitated, resuspended in TE buffer, and subjected to reverse transcription. The cDNA was PCR amplified, and the PCR–DNA was subjected to T7 in vitro transcription to yield the RNA pool for the next in vitro selection cycle. In cycles 3 and 4, the selection stringency was increased by adding wild-type RNA (50-fold excess; 100 μM) to the binding reaction as a specific competitor (modified from Bartel et al., 1991). Competitor RNA was not amplified during selection because its flanking regions differ from the primer sets used for pool amplification. After four selection rounds the enriched pool was cloned and sequenced. Different restriction endonuclease sites were introduced by PCR. A new 5′ primer (5′-TCT AAT ACG ACT CAC TAT AGG Gct gca gAG TCC TCG CTC A-3′) contained a *Pst*I cleavage site, whereas the 3′ selection primer (5′-GTC gga tcc GAG TCG CGC GT-3′) already included a *Bam*HI restriction site. The PCR products were digested with the appropriate restriction endonucleases and cloned in the pUC19 plasmid vector (NEB). After transformation, 100 independent clones were isolated and sequenced following the standard dideoxy sequencing protocol.

Determination of binding ratios

The progress of the selection was followed using polyacrylamide-urea gels under denaturing conditions. Pool RNA (40 nM) was incubated with 10 nM of the SelB protein in the presence of 200-nM wild-type 60.2 RNA as a specific competitor. Assays were performed in binding buffer in the presence of 400 U/mL RNAsin. After 1 h at 37 °C, the incubation mixtures were filtered over nitrocellulose filters; the bound RNA was eluted as described above and loaded onto a denaturing polyacrylamide gel (modified from Bartel et al., 1991). The same procedure was performed for measurements of the binding ratio of individual aptamers.

Filter-binding assays

Affinity of selected RNA molecules for SelB was determined by nitrocellulose filter-binding assays as described previously (Klug et al., 1997).

Gel-shift assays

The selected aptamers were tested for binding to three different proteins in native gel-shift experiments. Individual RNA sequences (75 nM) were incubated with various concentrations of protein in selection buffer for 1 h at 37 °C. Samples were placed on ice and, after the addition of 5% glycerol, loaded on 5% polyacrylamide gels under nondenaturing conditions. Gel electrophoresis was performed at 4 °C at 50 V.

Chemical and enzymatic probing

Chemical probing (Stern et al., 1988) of free RNA or RNA–SelB complexes was performed as described previously (Hüttenhofer et al., 1996). Enzymatic probing was carried out with S1 nuclease in 50 mM sodium cacodylate, pH 6.5, or 50 mM Tris-HCl, pH 7.2, and 10 mM $MgCl_2$ for 5 min at 20 °C. Reactions were terminated by the addition of 2.5 μg carrier tRNA and 0.3 M sodium acetate (pH 6.2), followed by two phenol and one chloroform extraction; subsequently, samples were ethanol precipitated and redissolved in 5 μL H_2O.

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