cytoplasmic tyrosine residues. Stimulation of human umbilical vein endothelial cells with VEGF-VPF results in tyrosine phosphorylation of a protein with a molecular size of approximately 200 kD (*21*) and mobilization of calcium (*26*). However, we did not observe a ligand-dependent increase in tyrosine phosphorylation of Flt in oocytes (*22*). The *flt* protein expressed in *Xenopus* oocytes is constitutively phosphorylated on tyrosine residues, so it may be difficult to detect ligand-induced phosphorylation.

High-affinity binding sites for VEGF-VPF have a tissue distribution similar to that of *flt* mRNA (*15, 27*). The *flt* transcript was detected in human umbilical vein endothelial cells that respond to VEGF-VPF (*28*). However, it is not known whether Flt is the only receptor for VEGF-VPF or whether it mediates the change in endothelial cell growth and vascular permeability induced by VEGF-VPF. Other endothelial cell mitogens, such as FGF, do not affect endothelial cell permeability or monocyte migration, suggesting that there are unique signal transduction pathways activated by VEGF-VPF.

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17. Human placental polyadenylated RNA was specifically primed with an oligonucleotide hybridizing to the *flt* 3′ untranslated sequence (nucleotides 4288 to 4313). A Sal I restriction site was added to the oligonucleotide (5′GATGTCGACGGTATAAATA CACATGTGCTTCTAG). Subsequently, a PCR was performed with the same primer and an oligonucleotide containing the unique Bgl II site in *flt* (nucleotides 3233 to 3248) (5′CTATGGAAGATCTGATT-TCTTACAGT). The generated fragment was digested with Bgl II and Sal I and ligated with the 5′ fragment of clone 9 (Eco RI–Bgl II) in the expression vector pSV7d (Eco RI–Sal I).
18. The *flt* sequence encoding the transmembrane domain was isolated from human placental mRNA by priming of a reverse transcriptase reaction with an oligonucleotide complementary to nucleotides 2801 to 2828 (5′CACAGTCCGGCACGTAGGTGATT). To start the PCR, an oligonucleotide that bound upstream of the transmembrane encoding sequence (nucleotides 2411 to 2435) (5′GTCACAGAAGAGGAT-GAAGGTGTCTA) was added. The generated 417-bp fragment was purified from an agarose gel and ligated in M13mp18 linearized with Sma I. The sequence was determined by the dideoxy chain termination method.
19. We transiently transfected COS cells by the DEAE-dextran method. Briefly, 7×10^6 cells in a 10-cm plate were incubated for 3 hours with 5 ml of Dulbecco's modified essential medium (DMEM), 2.5% fetal calf serum, 0.4 mg DEAE dextran per milliliter, 0.1 mM chloroquine phosphate, and 1 μg of expression plasmid per milliliter. This transfection medium was then replaced by DMEM containing fetal calf serum (10%). Binding experiments were performed after 72 hours.
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25. Fully grown oocytes (Dumont stage VI) were obtained from *Xenopus laevis*. The oocytes were defolliculated with collagenase (Sigma type IA, 1 mg/ml) and maintained at 19°C in modified Barth's medium (MBS) with Hepes (15 mM, pH 7.6), bovine serum albumin (1 mg/ml), penicillin G (100 U/ml), and streptomycin (100 μg/ml). The oocytes were injected with 50 nl of RNA solution or pure water (*23, 24*).
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29. The human recombinant 165–amino acid species of VPF-VEGF was expressed in Chinese hamster ovary cells (*9*) and purified to homogeneity as described [N. Ferrara, D. W. Leung, G. Cachienes, W. J. Winer, W. J. Henzel, *Methods Enzymol.* **198**, 391 (1991)]. VPF-VEGF was iodinated by an indirect iodogen method (*27*). The radioligand was tested for biological activity in an endothelial cell proliferation assay [D. Gospodarowicz, S. Massoglia, J. Cheng, D. K. Fuji, *J. Cell Physiol.* **127**, 121 (1986)].
30. Lysis buffer: 20 mM tris (pH 8.0) 137 mM NaCl, Triton X-100 (1%, v/v), glycerol (10%, v/v), 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) aprotinin (0.15 U/ml), 0.02 mM Leupeptin.
31. The $^{45}Ca^{2+}$ efflux from injected *Xenopus* oocytes was quantitated (*23, 24*). Two days after injection, the oocytes were incubated with $^{45}Ca^{2+}$ (100 μCi/ml) in 0.5 ml of Ca^{2+}-free MBS for 3 hours at 19°C. The oocytes were then washed with MBS and groups of eight oocytes were placed in 24-well plastic tissue culture plates. The medium was collected and replaced every 10 min, and the radioactivity in the medium was determined by liquid scintillation counting. After 30 min VEGF-VPF (20 ng/ml) was added to the medium for the first 10 min only. These experiments were performed at room temperature.

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Vegetal Messenger RNA Localization Directed by a 340-nt RNA Sequence Element in *Xenopus* Oocytes

KIMBERLY L. MOWRY AND DOUGLAS A. MELTON

Contained within a single cell, the fertilized egg, is information that will ultimately specify the entire organism. During early embryonic cleavages, cells acquire distinct fates and their differences in developmental potential might be explained by localization of informational molecules in the egg. The mechanisms by which Vg1 RNA, a maternal mRNA, is translocated to the vegetal pole of *Xenopus* oocytes may indicate how developmental signals are localized. Data presented here show that a 340-nucleotide localization signal present in the 3′ untranslated region of Vg1 RNA is sufficient to direct RNA localization to the vegetal pole.

THE BASIS FOR PATTERN FORMATION in early development can be traced to the localization of maternal factors in eggs. Although there is evidence for the existence of localized cytoplasmic determinants in a number of systems (*1*), in no case is the localization process understood. This localized information can in principle be stored as RNA or protein. In vertebrate eggs one example of this phenomenon is Vg1 RNA, a maternal RNA found exclusively in the vegetal hemisphere of *Xenopus laevis* oocytes and eggs (*2*). Vg1 RNA codes for a protein that is homologous to the transforming growth factor β (TGF-β) (*3*), but its role in embryogenesis is not currently understood. Vg1 RNA is evenly distributed in young oocytes but is translocated later, during the vitellogenic phase of oogenesis, to the vegetal hemisphere in mature oocytes and unfertilized eggs (*4*). After fertilization, Vg1 RNA, and ultimately Vg1 protein, are inherited preferentially by the vegetal blas-

Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138.

tomeres (*3, 5*). The localization phenomenon can be mimicked in culture; when immature oocytes are cultured appropriately (*6*), endogenous Vg1 RNA becomes vegetally localized, as does injected Vg1 RNA generated by in vitro transcription of a nontranslatable Vg1 cDNA clone. The ability of synthetic Vg1 RNA to be localized after injection rules out possible models for Vg1 RNA localization that involve transcription or translation of Vg1 itself and suggests that there are signals contained within the Vg1 RNA which mediate localization.

To map a putative localization signal on Vg1 RNA, we first tested whether specific portions of Vg1 RNA are necessary for vegetal localization, focusing initially on the 3′ untranslated region (UTR) of Vg1 RNA that comprises over half of the mRNA. Immature oocytes were injected with synthetic RNA transcripts, and localization was monitored by ribonuclease (RNase) protection assays of RNA obtained from animal (A) or vegetal (V) halves of the injected oocytes. Immediately after injecting them (0 days), we harvested a small number of oocytes to compare injected RNA levels before and after culture as a control for RNA degradation. Because the RNA was injected without regard to any A-V position (the A-V axis is not readily apparent in young oocytes) these assays also showed that the injected RNA was not consistently deposited at the vegetal pole, but randomly distributed. The remaining injected oocytes were cultured for 5 days during which Vg1 RNA can translocate to the vegetal pole and RNA not specifically localized will diffuse throughout the oocyte (*6*). RNA for injection was transcribed from either the entire 2.4-kb Vg1 cDNA or the 1.1-kb coding region alone (Fig. 1A). The full-length injected Vg1 RNA localized to the vegetal hemisphere, as did the endogenous Vg1 mRNA. Notably, Vg1 RNA molecules lacking the 3′ UTR (Vg1Δ3′UTR) were not localized in culture, whereas the endogenous Vg1 mRNA in the same oocytes was properly localized, suggesting that the 3′ UTR is necessary for localization.

To determine whether the Vg1 3′ UTR is also sufficient to specify localization, we tested the ability of Vg1 RNA sequences to direct the localization of *Xenopus* β-globin (XβG) RNA, which is normally not present in oocytes and does not itself localize (Fig. 1B). Chimeric constructs in which portions of the Vg1 cDNA were positioned downstream of the β-globin coding region were used in vitro to generate RNA transcripts for injection. While the injected XβG RNA was not localized, the injected chimeric (XβG-Vg3′UTR) RNA was localized almost exclusively to the vegetal half, indicating that the 1.3-kb Vg1 3′ UTR can direct vegetal localization when fused to β-globin sequences.

When smaller fragments from the 3′ UTR were tested (Fig. 2), the smallest restriction fragment found competent to direct vegetal localization of XβG sequences was 366 nucleotides (nt). In situ hybridization was used to determine whether the chimeric RNA containing a 366-nt sequence exhibited a subcortical localization similar to that of endogenous Vg1 RNA. The injected XβG-366 RNA localized to the vegetal pole in a pattern strikingly similar to that of endogenous Vg1 RNA (Fig. 3).

To define a minimal RNA localization element, we used the 366-bp fragment as a substrate for nuclease deletions from both the 5′ and 3′ ends. Deletion constructs (Fig. 2) were tested for localization with the use of in vitro–transcribed RNA (Fig. 4A). As determined by the RNase protection assay (Fig. 4A) and in situ hybridizations (*7*), deletion of 26 nt from the 3′ end of the 366-nt element did not impair its ability to confer localization on β-globin sequences. Removal of an additional 36 nt at either the 3′ or 5′ end, however, essentially eliminated localization.

To test whether only sequences at both ends of the 340-nt element are important for localization, we generated constructs lacking sequences from the middle of the element (Fig. 2). For construct XβG-340Δ+, the middle of the element was replaced by an equivalent length of XβG coding sequence, which is inert for localization. Only constructs containing the intact 340-nt element were capable of directing localization (Fig. 4B).

Comparison of the 340-nt sequence with

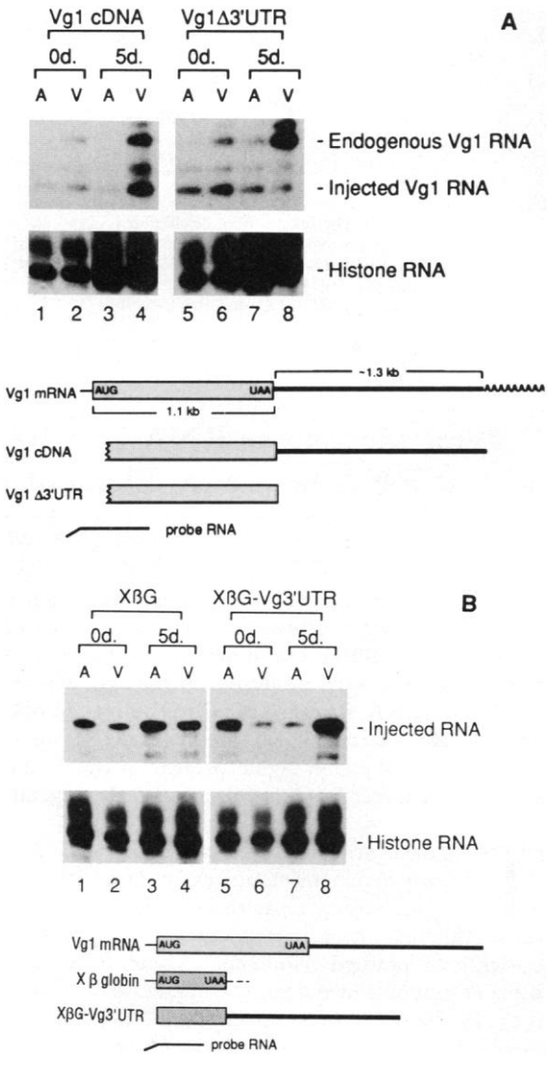

Fig. 1. The 3′ UTR of Vg1 RNA is necessary and sufficient to direct RNA localization. (**A**) An RNase protection assay was used to test for sequences involved in localization. Immature oocytes [stages III to IV (*15*)] were injected with RNAs transcribed in vitro by SP6 polymerase (*16*) from a 5′ foreshortened 2.4-kb Vg1 cDNA (*3*) which had been linearized either at the 3′ end of the insert to generate full-length transcripts (Vg1 cDNA, lanes 1 through 4) or at a Bst EII site at the end of the coding sequence, yielding transcripts lacking the 3′ UTR (Vg1Δ3′UTR, lanes 5 through 8). After culture for 4 to 5 days (*6*), oocytes were fixed (95% ethanol, 5% acetic acid) and cut into A and V halves. RNA was extracted (*17*) from the A and V halves either immediately after injection (0 days, lanes 1, 2, 5, and 6) or after culture to allow localization (5 days, lanes 3, 4, 7, and 8), and RNase protection was performed (*16*). RNA samples were probed with a 5′ Vg1 probe (spanning nucleotides 1 to 399) which can distinguish between the longer endogenous mRNA, giving a protected fragment of ~400 nt (indicated at the right as endogenous Vg1 RNA) and the 5′ foreshortened injected RNA which generated a protected fragment of ~340 nt (designated at the right as injected Vg1 RNA). To ensure that the differences in distribution of Vg1 RNA after culture were not due to unequal RNA recovery, RNA samples were also probed for histone H4 RNA, which is uniformly distributed in oocytes (*18*). The RNA fragments protected by the H4 probe are indicated at the right as histone RNA. (**B**) Immature oocytes, as above, were injected with RNAs transcribed from either a construct containing the XβG (*19*) coding sequence (lanes 1 through 4) or a chimeric construct (XβG-Vg3′UTR) in which the β-globin coding sequence had been fused to the Vg1 3′ UTR (lanes 5 through 8). As in (A), for RNase protection assays samples were probed with a 5′ β-globin probe (spanning nucleotides 1 to 323) to detect the injected RNAs (*20*).

sequences of other RNAs known to be localized during oogenesis, namely An1, An2, and An3 from *Xenopus* (*2*), as well as the *Drosophila* maternal transcripts *bicoid*, *nanos*, and *oskar* (*8, 9*), revealed no significant homologies. Both Vg1 RNA and *bicoid* RNA contain sequences specifying localization in their 3′ UTRs; the *bicoid* localization domain maps to a 625-nt segment in the 3′ UTR (*10*). However, Vg1 and *bicoid* RNA localization are not likely to be the same process. Vg1 RNA is initially distributed uniformly throughout the oocyte cytoplasm and is later translocated to the vegetal pole (*4*). In contrast, *bicoid* RNA enters the *Drosophila* oocyte at the anterior tip and is retained there (*11*). Both *nanos* and *oskar* RNA also enter the oocyte at the anterior tip but then are (like Vg1) translocated across the oocyte to the posterior pole (*9*). Thus, it is perhaps more likely that parallels will be found between Vg1 and *nanos* or *oskar* RNA localization.

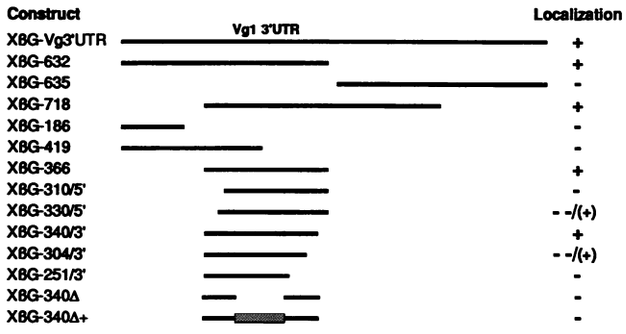

Fig. 2. Constructs tested for RNA localization. Using standard cloning procedures (*21*), we constructed chimeric clones containing β-globin and Vg1 sequences by fusing portions of the Vg1 3′ UTR (whose relative positions in the sequence are shown) to the Bst EII site at the end of the β-globin coding sequence. Nuclease Bal 31 was used (*21*) to generate deletions from the 5′ and 3′ ends of a 366-bp Ssp I–Bsm I fragment from the Vg1 3′ UTR. The chimeric deletion constructs, designated as /5′ and /3′, were created by fusion of the deleted fragments to β-globin coding sequences, as above. Constructs with deletions within the 340-nt element were prepared by deletion of 150 bp from the middle of the 340-bp sequence, leaving 90 bp from the 5′ end of the element and 100 bp from the 3′ end. The ends were religated directly to generate the construct XβG-340Δ, or the deleted 150 bp of Vg1 sequence was replaced by 150 bp of the XβG sequence to give the construct XβG-340Δ+. Names of the constructs are listed to the left; XβG refers to the *Xenopus* β-globin coding sequence, and the numbers indicate the length (in base pairs) of the added Vg1 segment. Thick lines, Vg1 3′ UTR; shaded box, β-globin coding sequence. The results of localization assays are summarized at the right: (+) reproducible localization, (−) no detectable localization, and [−−/(+)] irreproducible and almost undetectable localization (*22*).

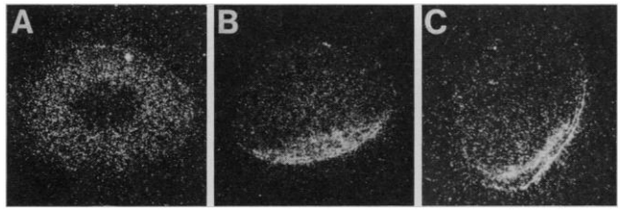

Fig. 3. In situ hybridizations to detect localized RNAs. Oocyte sections are shown with the vegetal pole at the bottom, and the hybridization signal appears as white grains. (**A**) Oocyte injected with RNA transcribed from the XβG construct and hybridized (*4*) with an XβG probe. Hybridization is uniform throughout the cytoplasm and the germinal vesicle is evident as a non-hybridizing "hole" at the center. (**B**) Oocyte injected with RNA transcribed from the chimeric construct (XβG-366) and hybridized with an XβG probe. Hybridization to the injected RNA is localized to the vegetal hemisphere. (**C**) Oocyte hybridized with a Vg1 probe to detect endogenous Vg1 RNA. The hybridization signal is restricted to the vegetal hemisphere.

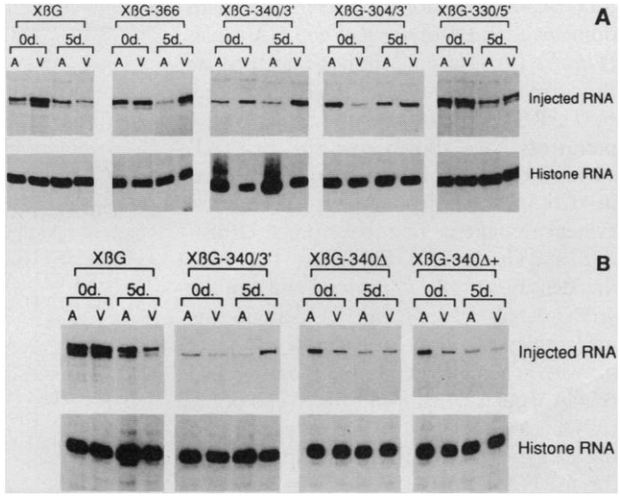

Fig. 4. RNA transcribed from the chimeric deletion constructs (Fig. 2) tested for localization. Samples were probed with a 5′ β-globin probe to detect the injected RNAs and a histone H4 probe to control for RNA recovery as described (Fig. 1). (**A**) Oocytes were injected with RNAs transcribed from the constructs indicated. (**B**) RNAs transcribed from the designated constructs were injected into oocytes. A, animal hemisphere; V, vegetal hemisphere; 0d., no culture after injection; 5d., 5-day culture after injection.

The Vg1 localization element, as large as 340 nt, may consist of several binding sites for localization factors. Recognition of RNA by proteins often involves elements of secondary structure rather than (or in addition to) a primary sequence. Thus, it is possible that a specific secondary structure, involving long-range pairings of sequences in the element, might be required for recognition of the RNA molecule by localization factors. For example, conservation of a predicted secondary structure rather than a primary sequence has been observed for the 625-nt localization domain on *bicoid* RNA (*12*). No structural similarities were apparent, however, when computer-generated RNA secondary structures for the 340-nt Vg1 localization element were compared to the predicted secondary structure (*12*) of the *bicoid* localization element or to computer-generated secondary structures for either *nanos* or *oskar* RNA. These computer-generated secondary structures can help direct further studies, but it is clear that experimental tests are necessary to determine whether these structures are assumed by localization elements in vivo.

In addition, the cytoskeleton has been implicated in Vg1 localization (*13, 14*). A two-step model for Vg1 localization has been proposed (*14*) whereby microtubule-mediated translocation to the vegetal hemisphere is followed by microfilament-dependent anchoring at the oocyte cortex. Cytoskeletal elements might bind directly to Vg1 RNA, or, perhaps more likely, such an association might be mediated by other components that associate directly with Vg1 sequences.

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20. The distribution of the injected XβG-Vg3′UTR RNA in the animal and vegetal hemispheres at day 0 (Fig. 1B, lanes 5 and 6) is a random occurrence due to the relatively small pool of oocytes assayed for that time. In addition, the assay results after culture for a single experiment should not be taken to provide a quantitative assessment of the ability of a construct to direct localization but rather are representative of the results obtained for a particular sequence. The extent of localization seen in this experiment was significantly greater than was routinely obtained, although in every case localization was observed.
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22. Reproducible localization was assigned only to those constructs that were consistently localized in the multiple pools of oocytes assayed after culture in each experiment. In side-by-side comparisons, the constructs XβG-366 and XβG-340/3′ were localized as much or more than construct XβG-Vg3′UTR.
23. We thank the members of the Melton laboratory for helpful advice and discussions, P. Klein and J. Clifton for comments on the manuscript, and J. Yisraeli both for providing the XβG-Vg3′UTR construct and for instruction on the oocyte culture system. K.L.M. is a fellow of the Helen Hay Whitney Foundation. Supported by grant GM 32921 from the NIH.

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The Specificity of Translational Control Switched with Transfer RNA Identity Rules

M. GRAFFE, J. DONDON, J. CAILLET, P. ROMBY, C. EHRESMANN, B. EHRESMANN, M. SPRINGER*

The interaction of *Escherichia coli* threonyl–transfer RNA (tRNA) synthetase with the leader sequence of its own messenger RNA inhibits ribosome binding, resulting in negative translational feedback regulation. The leader sequence resembles the substrate ($tRNA^{Thr}$) of the enzyme, and the nucleotides that mediate the correct recognition of the leader and the tRNA may be the same. A mutation suggested by tRNA identity rules that switches the resemblance of the leader sequence from $tRNA^{Thr}$ to $tRNA^{Met}$ causes the translation of the threonyl-tRNA synthetase messenger RNA to become regulated by methionyl-tRNA synthetase. This identity swap in the leader messenger RNA indicates that tRNA identity rules may be extended to interactions of synthetases with other RNAs.

THE EXPRESSION OF THE GENE FOR *E. coli* threonyl-tRNA synthetase, *thrS*, is negatively autoregulated at the translational level (*1, 2*). The threonyl-tRNA synthetase (ThrRS) binds to the region of the *thrS* mRNA leader that has structural analogies with the natural substrate ($tRNA^{Thr}$) of the enzyme (*3, 4*). The synthetase binds preferentially to tRNA and, if the cellular concentration of the primary substrate decreases, secondarily to the mRNA (*5*). This regulatory scheme is reminiscent of that of the translational feedback mechanism of ribosomal proteins (*6*). We use tRNA identity rules (*7*) to show that ThrRS interacts with stable RNA and mRNA in a similar fashion.

The *thrS* mRNA leader (Fig. 1) is composed of four domains (*4*). Mutational analysis indicated that only domains 2 and 4 are directly involved in the control of *thrS* expression (*8*). These two domains bind directly to the synthetase and compete with $tRNA^{Thr}$ for this binding (*5*). Therefore, we compared the consensus structure of the four $tRNA^{Thr}$ isoacceptors of *E. coli* with domains 2 and 4 of the *thrS* mRNA leader (Fig. 1) (*9*). The nucleotides that mediate the correct recognition of the isoacceptor set by ThrRS define the identity elements. Experiments have shown that the BGU (B stands for C, G, or U) anticodon is involved in $tRNA^{Thr}$ identity (*10, 11*). Theoretical evidence suggests that base pairs U(68)-A(5) and G(71)-C(2) (Fig. 1) also belong to the identity set (*12*). Domains 2 and 4 of the *thrS* mRNA leader (Fig. 1) contain the nucleotides that appear to define $tRNA^{Thr}$ identity at equivalent places. Moreover, an ACCA sequence that forms the 3′ end of the tRNA is located at an equivalent position in the leader mRNA. Therefore, it appears that the mRNA leader of *thrS* might have the identity of $tRNA^{Thr}$. We postulated that the identity of the mRNA leader could be changed from $tRNA^{Thr}$ to that of another tRNA, causing control of the *thrS* gene to be dependent on another synthetase.

We decided to change the CGU ($tRNA^{Thr}$ anticodon) to CAU ($tRNA^{Met}$ anticodon) in the mRNA leader equivalent of the anticodon for the following reasons. (i) The principal identity element of both tRNAs appears to be the anticodon. (ii) Clones that overproduce methionyl-tRNA synthetase (MetRS) were available (*13*). (iii) We have isolated the desired mutation as a constitutive mutant that has no effect on the structure of the mRNA leader (*3, 4*).

We introduced a second mutation in the ribosomal binding site (RBS) of the *thrS* mRNA leader. Although this second mutation was not involved in the identity change, we introduced it to increase the sensitivity of *thrS* expression to regulation. The RBS is indirectly involved in control of *thrS* expression because the synthetase competes with the ribosome for binding to the mRNA (*5*). Thus, if the affinity of the ribosome for the mRNA is lowered because of an RBS mutation, the synthetase binding to the mRNA is not inhibited, allowing regulation over a greater range. Conversely, if the affinity of the ribosome for the RBS is increased, a concomitant decrease in regulation is observed (*8*). Therefore, any effect of MetRS

Table 1. The effect of *thrS* mRNA leader mutations on the repression caused by ThrRS (plasmid pUB4) or MetRS (plasmid pNAV7) compared to a pUC12 control plasmid. The β-galactosidase values expressed from the fusions are given in Miller units per absorbance at 650 nm (A_{650}) of bacteria (*19*) and are the averages ± the standard deviation of at least four measurements made between 0.2 and 0.4 A_{650}. The *thrS-lacZ* fusions are indicated as the name of the λ bacteriophages that carry them. The sequence of the mRNA leader counterpart of the anticodon and the corresponding amino acid are given next to the name of the fusion.

thrS-lacZ fusion	Expression of β-galactosidase		
	pUC12	pUB4	pNAV7
λMΔ20-10-XII-25 CGU (Thr)	105 ± 5	17.2 ± 1.3	45 ± 9
λMΔ20-10-XII-25-BS4-9 CAU (Met)	1534 ± 106	1462 ± 97	130 ± 6
λMΔ20-10-XII-25-VII-5 CCU (Arg)	1322 ± 91	1190 ± 79	1475 ± 181
λMΔ20-10-XII-25-VI-1 CUA (Amber)	1757 ± 56	1611 ± 83	1752 ± 20
λMΔ20-10-XII-25-JC4-2 UUG (Gln)	1706 ± 73	1557 ± 39	1890 ± 293
λMΔ20-10-XII-25-ML2 Δ(−21 to −39)	3254 ± 123	3130 ± 153	3107 ± 362

M. Graffe, J. Dondon, J. Caillet, M. Springer, Institut de Biologie Physico-Chimique, 75005 Paris, France.
P. Romby, C. Ehresmann, B. Ehresmann, Institut de Biologie Moléculaire et Cellulaire, 67084 Strasbourg, France.

*To whom correspondence should be addressed.