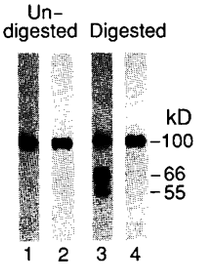

Fig. 3. RGD–affinity chromatography of platelets incubated in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of chymotrypsin. After incubation, the platelets were solubilized in octylglucoside, and applied to an RGD–affinity column (*3*). The flow-through (lanes 1 and 3) and the bound (lanes 2 and 4) materials were analyzed on SDS-PAGE (7% gels, nonreducing conditions), transferred to PVDF membranes, and then probed with a monoclonal antibody to gpIIIa.

motrypsin such that approximately 50% of the gpIIIa was degraded to the 66-kD derivative. The digested and control (undigested) platelets were then solubilized and passed over RGD affinity columns (*3*). After washing to obtain a flow-through fraction, the columns were eluted with free RGD peptide. The flow-through and the eluate were subjected to SDS-PAGE, transferred, and immunoblotted with a monoclonal antibody (MAb) to gpIIIa. Intact gpIIIa was observed in the flow-through from the undigested platelets, and a mixture of intact gpIIIa and the 66-kD gpIIIa fragment was present in the flow-through from the chymotrypsin-treated platelets (Fig. 3). In the RGD eluates from both columns, only intact gpIIIa was detected. Thus, the 66-kD fragment was not retained on the RGD affinity column. This result is compatible with interpretation that the site to which RGD peptides bind and to which they crosslink coincide and reside in the NH_2-terminal aspects of gpIIIa.

In sum, our results indicate that the lysine residues within gpIIIa 109–170, particularly Lys^{125}, reside in close proximity to the lysine in Fn-7 when it interacts with the RGD binding site in gpIIb/IIIa. In addition, the NH_2-terminal region of gpIIIa may be involved in the function of this adhesion receptor; and, on the basis of homology, the related sequences in the β subunits of the other integrins may also be involved in their adhesive functions. This is also consistent with the inhibition of platelet aggregation by a MAb to the NH_2-terminal region of gpIIIa (*11*). It must be emphasized, however, that the identified region resides in close proximity but may not be the RGD binding site. In fact, the RGD cross-linking site on gpIIIa probably does not constitute the complete recognition site or sites involved in the binding of adhesive proteins to gpIIb/IIIa or other integrins. Clearly, the α subunits play an important role in regulating the specificity of the integrins (*12*), and it is likely that the recognition site contains elements contributed by both subunits. The localization of the gpIIb region to which RGD and fibrinogen γ chain peptides crosslink (*7, 8*) may provide a means of identifying the sequences within an α subunit that may also contribute to the function of this family of adhesion receptors.

Note added in proof: With a different peptide and cross-linker, Smith and Cheresh (*13*) have identified a similar region (61–203 of the β subunit) in a vitronectin receptor from placenta involved in cross-linking.

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$tRNA_i^{met}$ Functions in Directing the Scanning Ribosome to the Start Site of Translation

A. MARK CIGAN, LAN FENG, THOMAS F. DONAHUE

The mechanism by which the scanning ribosome recognizes the first AUG codon nearest the 5′ end of eukaryotic messenger RNA has not been established. To investigate this an anticodon change (3′-UCC-5′) was introduced into one of the four methionine initiator ($tRNA_i^{met}$) genes of *Saccharomyces cerevisiae*. The ability of the mutant transfer RNA to restore growth properties to *his4* initiator codon mutant yeast strains in the absence of histidine was then assayed. Only the complementary codon, AGG, at the *his4* initiator region supported His^+ growth. The mutant transfer RNA also directed the ribosome to initiate at an AGG placed in the upstream region of the *his4* message. Initiation at this upstream AGG precluded initiation at a downstream AGG in accordance with the "scanning" model. Therefore, an anticodon:codon interaction between $tRNA_i^{met}$ as part of the scanning ribosome and the first AUG must function in directing the ribosome to the eukaryotic initiator region.

A FUNDAMENTAL DIFFERENCE between prokaryotic and eukaryotic organisms is the mechanism of initiation of protein synthesis. In prokaryotes, the 30*S* ribosomal subunit binds the start site of translation in mRNA (*1*). This recognition process is predominantly mediated by the 3′ end of 16*S* ribosomal RNA, a component of the 30*S* subunit, which base pairs with a complementary ribosomal binding site (Shine/Dalgarno sequence) that is located 5′ to a start codon. In eukaryotes, ribosomal binding sites of the prokaryotic type are not present in mRNA (*2*). Instead, the ribosomal scanning model (*3*) proposes that the 40*S* ribosomal subunit in association with methionine initiator tRNA ($tRNA_i^{met}$)

Department of Molecular Biology, Northwestern University Medical School, Chicago, IL 60611.

and initiation factors binds the "capped" 5′ end of the message and migrates in a 5′ to 3′ direction, "scanning" for a translational start site. Therefore, in the absence of ribosomal binding sites in eukaryotic mRNA, the predominance of the first AUG codon serving as the start site of translation in mRNA has suggested that the AUG codon itself might serve as a basic signal for ribosomal recognition of the initiator region (*3*).

Although the ribosomal scanning model is based on sound experimental evidence, it remains unclear which components of the preinitiation complex function in establishing the start site of translation. One apparent difference between the prokaryotic and the eukaryotic preinitiation complex is that eukaryotic methionine initiator tRNA is associated with the ribosome before it binds to mRNA (*4*). In light of the predominance of the first AUG "signal," this association of $tRNA_i^{met}$ with the 40*S* subunit could imply a mechanistic requirement for ribosomal recognition of an AUG start codon. Perhaps in contrast to a ribosomal RNA (rRNA)/mRNA base pair interaction observed for prokaryotes, a base pair interaction between the anticodon of $tRNA_i^{met}$, as part of the scanning ribosome and the first AUG triplet in mRNA, may mediate ribosomal recognition of the eukaryotic initiator region.

To address the role of $tRNA_i^{met}$ during the translation initiation process, we isolated three of the four $tRNA_i^{met}$ genes from the haploid genome of the simple eukaryotic yeast, *Saccharomyces cerevisiae* (*5*), and constructed all nine possible point mutations of the AUG start codon at the *HIS4* locus (*6*).

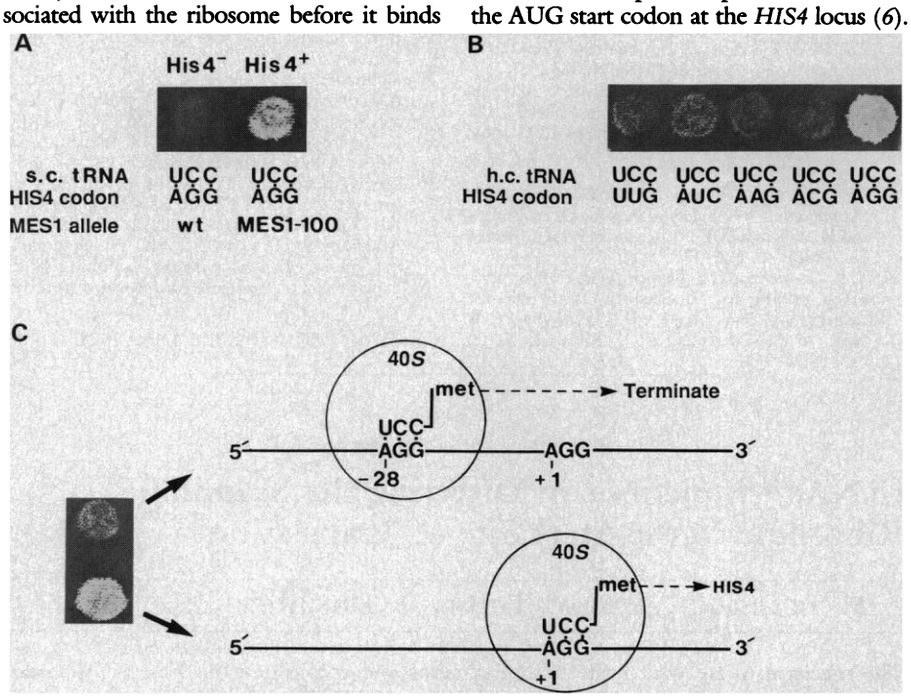

Fig. 1. Growth properties (on SD medium minus histidine) of *his4* initiator codon mutant yeast strains containing a $tRNA_i^{met}$ gene with an anticodon mutation at position +35 (3′-UCC-5′). The mutant $tRNA_i^{met}$ gene was generated by site-directed mutagenesis (*7*). The mutant tRNA gene, contained on an 850-bp Hind III restriction fragment was subcloned into the Hind III site of the *URA3*$^+$ single-copy (s.c.) yeast vector YCp50 and YIp5 and 2-μm DNA from the yeast vector YEp24 was subcloned into the mutant tRNA YIp5-derived plasmid to generate a high-copy (h.c.) yeast vector. The single- or high-copy tRNA plasmids were used to transform (*27*) *his4* initiator codon mutant strains to *URA3*$^+$, purified and tested for their ability to grow on SD minus histidine plates (3 days at 30°C). Construction of initiator codon mutations at *HIS4* have been previously described (*6*). All yeast strains are isogenic to TD28 (*MAT***a** *ura3-52 ino1-13*), an ascospore derivative of S288C (*MAT*α), which has been used extensively to characterized *HIS4* transcription (*12, 28*) and translation (*6, 23*). A single transcriptional initiation point maps 60 nucleotides upstream from the AUG start codon, the first AUG in the *HIS4* message. (**A**) The mutant UCC-$tRNA_i^{met}$ gene present on the YCp50 plasmid, in a yeast strain containing the AGG initiator codon mutation at *his4* (His4$^-$) compared to a spontaneous His4$^+$ revertant that contains the *MES1-100* allele. Wt, wild type. (**B**) The mutant tRNA on the high-copy plasmid in yeast strains (His4$^-$) that contain non-AUG initiator codon mutations at *his4* (UUG, AUC, AAG, and ACG) compared to the isogenic yeast strain (His4$^+$) bearing the complementary AGG-*his4* allele. These mutant initiator codons are not present in the 5′ noncoding region of the *HIS4* message (*12*). The efficiency of *HIS4* expression in the latter AGG, UCC-$tRNA_i^{met}$ strain is estimated to be 16 to 20% of wild-type repressed levels (*29*). (**C**) The high-copy mutant tRNA present in a yeast strain (His4$^-$) that contains an out-of-frame AGG codon 28 nucleotides (−28) from the AGG mutation at the normal *HIS4* initiator codon (+1), compared to the isogenic strain, which contains only an AGG mutation at the *his4* initiator region (His4$^+$). The AGG codon at position −28 in the *his4* leader was introduced by site-directed mutagenesis (*30*). Interpretations of the initiation events at these two different mRNAs are shown schematically.

In this report we tested the ability of an anticodon mutation in one of these tRNA genes to mediate ribosomal recognition of the mutant *his4* initiator region in yeast. The strategy employed is based on the established properties of the *his4* initiator codon mutants (*6*). In the absence of this first AUG codon at *HIS4*, ribosomes bypass this region, which results in the inability to grow in the absence of histidine (His$^-$). Thus, the physiological relevance of an anticodon:codon interaction in establishing start site selection can be assessed by a simple growth test. If the anticodon of $tRNA_i^{met}$ functions in directing the ribosome to the start site, then an anticodon mutation should direct the ribosome to the complementary non-AUG codon at the *his4* initiator region as detected by a His$^+$ phenotype.

The mutant tRNA gene was constructed by oligonucleotide site-directed mutagenesis (*7*) changing the wild-type +35 adenine nucleotide (3′-UAC-5′) to cytosine (3′-UCC-5′) so that it could potentially base pair with a +2 change of the *HIS4* AUG initiator codon, AGG (Fig. 1). This specific tRNA mutation was selected for two reasons. First, in vitro modification of the +34 and +36 anticodon positions of the prokaryotic $tRNA_f^{met}$ (*N*-formyl-methionine) decreases the ability of these tRNAs to be aminoacylated by the *Escherichia coli* methionyl-tRNA synthetase by greater than three orders of magnitude (*8*). However, of the +35 changes, an A to C change (3′-UCC-5′) only reduces aminoacylation of this mutant tRNA by 50-fold. On the basis of similar enzymatic properties (*9*) and amino acid homologies (*10*) between the yeast and *E. coli* methionyl-tRNA synthetases, it was anticipated that a yeast initiator tRNA with the +35 anticodon change, 3′-UCC-5′ (referred to as UCC-$tRNA_i^{met}$), would be the best candidate among mutant tRNAs to be aminoacylated in vivo and therefore active during the translation initiation process (*11*). Second, the complementary AGG triplet codon does not appear in the 5′ noncoding region of the *HIS4* message (*12*). Therefore, initiation at an upstream AGG will not preclude the ability to detect initiation at an AGG codon present at the *his4* initiator region; a property anticipated based on first AUG recognition rules in yeast (*6, 13*).

As shown in Fig. 1A, this mutant tRNA gene when present in yeast as part of the single-copy yeast vector YCp50 (*14*) cannot restore *HIS4* expression despite a complementary codon at the normal initiator region. However, upon prolonged incubation, this His$^-$ yeast strain reverts to His$^+$. Forty of these revertants were characterized by vector loss and subsequent retransformation with UCC-$tRNA_i^{met}$ (*15*). On the basis

of these studies, the His^+ phenotype associated with three of these revertants is dependent on the original UCC-$tRNA_i^{met}$, the complementary codon at *his4*, and a newly acquired chromosomal mutation. Genetic analysis showed that each of these three revertant strains contained a chromosomal mutation that was unlinked to *HIS4* and located on chromosome VII (*16*), being tightly linked to the previously mapped *MES1* gene, which encodes methionyl-tRNA synthetase (*17*).

Isolation of the *MES1* gene from one of the three His^+ revertants (Fig. 1A) and subsequent subcloning studies localized the mutation responsible for UCC-$tRNA_i^{met}$–dependent *HIS4* expression to a 1.0-kb Xba I DNA restriction fragment. This DNA fragment spans amino acid positions +311 to +639 in the previously described *MES1* DNA sequence (*10*). DNA sequencing of this entire 1.0-kb Xba I fragment in parallel with the fragment isolated from the isogenic parent strain identified a single-point mutation (GAT→*T*AT) that changes the aspartic acid at amino acid position +420 to a tyrosine (Fig. 2). This amino acid change is located 67 amino acids downstream from the $CysX_2CysX_9CysX_2Cys$ zinc(II) finger motif (*18*) and resides within a region that shows limited homology between the *E. coli* (*19*) and yeast (*10*) enzymes (Fig. 2). In light of the fact that *MES1* is a single-copy gene in yeast, this mutation in methionyl-tRNA synthetase (*MES1-100*) must confer the ability to "charge" the UCC-$tRNA_i^{met}$ (*20*) while maintaining the ability to aminoacylate the wild-type initiator and elongator Met-tRNA species.

The ability to detect a His^+ phenotype with these revertant strains strongly suggests that this mutant tRNA participates in ribosomal recognition and initiation at an AGG codon at *his4*, provided a charging defect conferred by the anticodon mutation is circumvented. As part of our analysis we realized that AGG *his4* strains containing the wild-type *MES1* gene were also His^+ when these strains contained the mutant UCC-$tRNA_i^{met}$ as part of a high-copy yeast vector (Fig. 1B). In addition, the same effect (His^+) could be obtained by maintaining the mutant tRNA in single copy and the wild-type *MES1* gene on a high-copy vector (*21*). Thus, by altering the dosage of mutant tRNA or the wild-type methionyl-tRNA synthetase, levels of aminoacylated mutant tRNA that can participate in the translation initiation process are increased.

Using the high-copy UCC-$tRNA_i^{met}$ expression system, we tested various aspects of the specificity of the anticodon:codon interaction in establishing the start site of translation. This system was employed as it affords an analysis in isogenic yeast strains. Therefore, differences in growth properties should be a direct reflection of simple base changes and their effects on the initiation process at *HIS4* as opposed to nonspecific effects on growth due to genetic background differences.

The UCC-$tRNA_i^{met}$ when present in yeast strains containing one of the noncomplementary initiator codon mutations AAG, ACG, AUC, or UUG at *his4* is incapable of restoring *HIS4* expression (Fig. 1B). This is in contrast to the His^+ phenotype observed when the complementary AGG codon is present at *his4* (Fig. 1B). This not only demonstrates the specificity of the mutant UCC-$tRNA_i^{met}$ for initiation at AGG but shows that a triplet codon other than AUG, namely AGG, can serve as the start site of translation provided the complementary anticodon is present in the initiator-tRNA.

We also tested the ability of UCC-$tRNA_i^{met}$ to mediate recognition of a complementary triplet in accordance with scanning rules established for AUG codon recognition in yeast (*6*). A *his4* allele was constructed by site-directed mutagenesis to now contain an AGG codon in the *his4* leader 28 nucleotides upstream and out-of-frame with the AGG codon present at the initiator region (Fig. 1C). When this construction is present at *HIS4* in yeast strains containing the UCC-$tRNA_i^{met}$ on a high-copy vector no *HIS4* expression can be detected (His^-; Fig. 1C). Similar results are obtained when AGG is placed 14 nucleotides upstream from a downstream in-frame AGG codon (*22*). Namely, this strain is His^- in contrast to the His^+ phenotype observed in the parent strain that does not contain the upstream AGG. In addition, fusion of this upstream AGG in the latter *HIS4* allele construction in-frame to *HIS4* results in a His^+ phenotype in the presence of the mutant tRNA, directly demonstrating that the upstream AGG serves as a site of initiation (*22*). In light of these observations and studies at *HIS4* (*23*), which show that deletions and point mutations in the *HIS4* leader have little or no effect on *HIS4* expression, we conclude that loss of *HIS4* expression is not a consequence of mutating the *his4* leader by introduction of an upstream AGG. Instead, the simplest explanation for the His^- phenotype in these strains is that it is a direct consequence of AGG

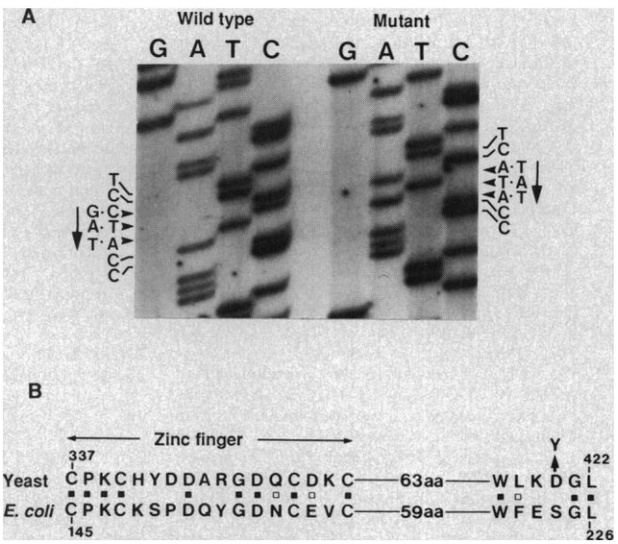

Fig. 2. DNA sequence analysis of wild-type *MES1* and the mutant *MES1-100* alleles. The DNA sequence of the wild-type *MES1* gene (methionyl-tRNA synthetase) has been previously described (*10*). The complete *MES1* gene is present on a 3.2-kb Eco RI–Bam HI restriction fragment capable of encoding a protein of 751 amino acids. The entire *MES1* region from the parent and single-copy UCC-$tRNA_i^{met}$ $His4^+$ revertant strain (Fig. 1A) was isolated by the integration/excision method (*31*) with a 5′ proximal 2.0-kb Hind III fragment that is contiguous with the *MES1* gene. To localize the mutation, DNA restriction fragments derived from the evicted mutant *MES1* gene were used to construct a series of functional chimeric *MES1* genes consisting of mutant and wild-type DNA restriction fragments. These chimeric genes were placed onto the single-copy *LEU2/CEN4* yeast vector and used to transform MC57-3B [*MAT***a**, *his4-318*(AGG), *ura3-52*(URA^+:UCC-$tRNA_i^{met}$) *leu2-3*, *mes1-1*, *ino1-13*] to $Leu2^+$. This strain contains a previously described temperature-sensitive allele of *MES1* (*mes1-1*) (*17*). The mutation in the *MES1-100* region was localized to the 1.0-kb Xba I DNA fragment by its ability to nonselectively confer a $His4^+$ phenotype to this strain in the presence of UCC-$tRNA_i^{met}$ as well as complement the temperature-sensitive phenotype of the *mes1-1* strain. The complete sequence of this fragment was determined by subcloning each of the wild-type and mutant *MES1* Xba I restriction fragments into mp10 and mp11. From a series of synthesized oligonucleotides complementary to the *MES1* coding region, the DNA sequence of both strands of the Xba I restriction fragment was determined. (**A**) The DNA sequence corresponding to the antisense strand of the wild-type and mutant *MES1-100* region (between amino acid positions +417 and +423). Labels for base pair regions indicate the normal and mutated codon for the wild-type and mutant *MES1-100* genes, respectively, and arrows indicate the direction of translation. (**B**) The position of the resulting amino acid change relative to the wild-type yeast *MES1* gene. A comparison of the yeast and *E. coli* amino acid sequences is also presented showing the zinc(II) finger domain and its relative position to the region containing the mutation in the *MES1-100* allele. Identical amino acids are shown as (■), conservative changes as (□).

being complementary to the anticodon of the mutant tRNA, which confers the ability to the ribosome to no longer bypass the *his4* leader but now recognize and initiate at the first AGG codon in the message (Fig. 1C). This codon is out-of-frame with the *HIS4* coding region. Therefore, as observed for upstream, out-of-frame AUG codons in yeast mRNAs (*6, 13*), recognition of this first AGG codon precludes the ability of the ribosome to continue to scan the leader region and initiate at the downstream AGG that would confer a His^+ phenotype (Fig. 1C).

Aside from providing a structure-function relation between $tRNA_i^{met}$ and the synthetase (Fig. 2), perhaps defining a specific interaction between the anticodon and a nucleic acid binding domain in the protein, our data illustrate two important features of the initiation process (Fig. 1). First, the anticodon of the $tRNA_i^{met}$ functions in directing the ribosome to initiate translation at the first complementary triplet codon in the message. Second, the ability to direct the ribosome to an AGG triplet located at two positions in the *his4* message, one of which does not correspond to a normal initiator region, suggests that sequence context is not essential to the recognition process in yeast. This would agree with genetic and mutational studies at *HIS4* that demonstrate that no simple sequence in the *HIS4* message can override the importance of an AUG in establishing the start site of translation (*6, 23*). Taken together, our analysis suggests that as the ribosome scans the 5′ noncoding region of mRNA the anticodon of $tRNA_i^{met}$, as part of this preinitiation complex, reads the mRNA. Once a complementary base pair interaction between the anticodon of tRNA and a triplet codon in mRNA is established, the ribosome then stops and the 80*S* initiation complex is assembled. Therefore, an anticodon:codon interaction between $tRNA_i^{met}$ and the first AUG in the message is a basic component for ribosomal recognition of the translational start site during the scanning process.

However, the observations that translation efficiency at an AUG codon can be varied by altering the sequence context (*23*), especially in higher eukaryotic mRNAs (*24*), suggests that ribosomal recognition of an initiator region may be more complex than a simple three base pair interaction between the anticodon of the $tRNA_i^{met}$ and the AUG. In agreement with this recent reversion, studies in our laboratory suggest that the eukaryotic initiation factor 2 (eIF-2) is also important for mediating start site selection. Specifically, mutations at a zinc(II) finger motif of the eIF-2β subunit confers the ability to the ribosome to initiate at a UUG codon in the absence of an AUG at *HIS4* (*25*). Interestingly, eIF-2 is intimately associated with $tRNA_i^{met}$ from the time of ternary complex formation (eIF-2·GTP·$tRNA_i^{met}$) up until the time of 80*S* complex formation, suggesting that both eIF-2 and $tRNA_i^{met}$ might work in concert in establishing the start site of translation. In light of similarities between the yeast and mammalian initiation process (*26*), these analyses provide insight into the general mechanism of translation initiation in virtually all eukaryotic organisms.

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15. His^+ revertant yeast strains were grown nonselectively on enriched medium (YEPD) for several generations, plated onto YEPD and replica plated to an SD minus uracil plate and an SD minus histidine plate. Ura^- strains that were still His^+, indicative of loss of the YCp50 vector and restoration of *HIS4* expression independent of the associated mutant tRNA, were not further characterized. Ura^-/His^- strains that became His^+ upon subsequent retransformation with the original vector containing the mutant tRNA were indicative of a chromosomal mutation resulting in *HIS4* expression that is dependent on UCC-$tRNA_i^{met}$ and were characterized genetically.
16. The Ura^-/His^- strains derived from the three His^+ revertants after vector loss experiments were retransformed [A. Hinnen, J. B. Hicks, G. R. Fink, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1929 (1978)] with the UCC-$tRNA_i^{met}$ as part of the vector YIp5 integrated at the *ura3-52* locus. These His^+ yeast strains, MC54-11A [*MATa his4-318*(AGG) *ino1-13 ura3-52*(Ura^+:UCC-$tRNA_i^{met}$),R12], MC55-11B [*MATa his4-318*(AGG) *inol-13 ura3-52*(Ura^+:UCC-$tRNA_i^{met}$),R39], and MC56-14C [*MATa his4-318*(AGG) *inol-13 ura3-52*(Ura^+:UCC-$tRNA_i^{met}$), R40] suspect of containing the chromosomal mutations at *MES1* (designated by R12, R39, or R40), were crossed to yeast strain MC57-4A [*MATα his4-318*(AGG) *ura3-52*(Ura^+:UCC-$tRNA_i^{met}$), *mes1-1*], which contains a temperature-sensitive allele (*mes1-1*) at the methionyl-tRNA synthetase locus (*17*). Tetrad analysis showed a $2^+:2^-$ segregation for growth on SD medium minus histidine plates. All His^+ meiotic products were temperature resistant, whereas all His^- meiotic products were temperature sensitive (*mes1-1*). This demonstrates that the chromosomal mutations responsible for the His^+ reversion events map to the *MES1* locus on chromosome VII.
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20. This *MES1-100* allele will not charge the UGC-$tRNA_i^{met}$ or the UUC-$tRNA_i^{met}$ as measured by our assay of *HIS4* expression. Thus, this mutant *MES1* is specific for the UCC-$tRNA_i^{met}$.
21. A. M. Cigan and T. F. Donahue, unpublished results.
22. A deletion of the normal initiator region between position −16 and +4 was made (*23*), and a unique Eco RI linker site (GGAATTCC) was inserted at the novel joint. At the 5′ end of the linker, an *AG*GAATTCC was fortuitously present in frame with the *HIS4* coding region. This *HIS4* allele when present in yeast along with the mutant tRNA expresses *HIS4* (growth on minus histidine media), thus this AGG is directly used for initiation. A complementary 23-bp oligonucleotide, 5′-AATTCTGAATA*AGG*GTTTGCCG-3′ with complementary Eco RI ends that contains an AGG initiator in the natural *HIS4* initiator sequence context (5′-AUA*AGG*GUU-3′) was then inserted at the Eco RI site; the *AG*GATTC at the Eco RI junction is now upstream and out-of-frame, whereas the downstream AGG within the oligonucleotide is in-frame. The mutant tRNA cannot restore *HIS4* expression to this allele in yeast. Thus initiation at the upstream out-of-frame AGG (−14 position), precludes initiation at the downstream in-frame AGG.
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29. We have quantitated the efficiency of *HIS4* expression by measuring β-galactosidase (β-gal) levels from yeast strains that contain the mutant tRNA on a high-copy vector as well as a proximal fusion of the AGG *his4* region to the *E. coli lacZ* coding region integrated at the *LEU2* locus on chromosome III. Control strains containing the wild-type tRNA on a high-copy plasmid have residual levels of β-gal approximately 0.4% of wild-type *HIS4-lacZ* strains [typical wild-type levels are 350 to 450 units, see (*6*)]. When the mutant tRNA is substituted this level is raised to 4% of wild-type *HIS4-lacZ* fusion control strains. In addition, the UCC-$tRNA_i^{met}$ did not increase the β-gal activity in yeast strains when the noncomplementary initiator codon mutations at *his4* were fused to *lacZ*. One difference between the β-gal and growth assay is that the strains in the latter assays are grown in the absence of histidine. As a result of poor *HIS4* expression, these strains will starve for histidine and derepress *HIS4* transcription four- to fivefold by the general amino acid control

system [T. F. Donahue, R. S. Daves, G. Lucchini, G. R. Fink, *Cell* **32**, 89 (1983)]. Thus, 4% levels of expression, as noted by the β-gal assay, will increase to 16 to 20% of repressed levels, which are sufficient for growth. This property has been established for other *his4* alleles and their corresponding *his4-lacZ* constructions (*23*).

30. An AGG codon was introduced 28 nucleotides upstream of the AGG codon at the +1 initiator region by site-directed mutagenesis with the oligonucleotide 5′-TAATACAAGGAATTCCAAAATTT-3′. The methods used for constructing the mutation, the DNA sequencing strategy to confirm the construction, and the subsequent *his4* allele construction have been previously described (*6, 23*). As a result of this mutagenesis the natural sequence in the *HIS4* leader region between positions −28 and −21, AUAGUUUA, was changed to AGGAAUUC.
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32. We thank R. Letsinger and K.-L. Ngai for their assistance with oligonucleotide synthesis, P. Sigler for oligonucleotides and helpful suggestions, G. R. Fink for the *mes1-1* strain, D. Garfinkel for the *LEU2/CEN4* vector, and S. Kowalczykowski, L. Lau, and R. Scarpulla for their critical review of the manuscript. This work was supported by USPHS grant GM32263 and in part by the Searle Scholars Program of the Chicago Community Trust awarded to T.F.D.

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Endothelial Adhesiveness for Blood Neutrophils Is Inhibited by Transforming Growth Factor–β

JENNIFER R. GAMBLE AND MATHEW A. VADAS

Adhesion of blood cells to endothelial cells is an essential component of all inflammatory responses. The capacity of the endothelium to support adhesion of neutrophils is increased by cytokines such as tumor necrosis factor–α, interleukin-1, and endotoxin. Another cytokine, transforming growth factor–β (TGF-β), was a strong inhibitor of basalneutrophil adhesion and also decreased the adhesive response of endothelial cells to tumor necrosis factor–α (TNF-α). The ability of cells to respond to TGF-β was related to the duration of culture of endothelial cells after explantation from umbilical veins. TGF-β is likely to serve an anti-inflammatory role at sites of blood vessel injury undergoing active endothelial regeneration.

ATTACHMENT TO THE ENDOTHELIUM is essential in the movement of cells from the circulation into the site of an inflammatory reaction. The level of cell attachment is markedly increased by cytokines such as TNF and interleukin-1 (IL-1), which also increase the levels of some molecules on the endothelial surface involved in adhesive reactions (*1–3*).

Endothelial cells also elaborate an extracellular matrix composed of glycoproteins such as laminin (*4*), fibronectin (*5*), and collagen (*6*) involved in cell anchorage and migration. Recently, TGF-β was shown to increase the incorporation of fibronectin and collagen into the extracellular matrix of fibroblasts, epithelial, and endothelial cells (*7, 8*). Furthermore, TGF-β also inhibits endothelial cell proliferation in response to growth-promoting factors (*9*) and wounding (*10*), which suggests that TGF-β may be an important regulator of function at the endothelial surface. We therefore examined the effect of this molecule on adhesive interactions involving endothelial cells and neutrophils.

Primary cultures of human umbilical vein (HUVE) endothelial cells exposed to TGF-β for 24 hours showed a reduced capacity to support neutrophil binding (Fig. 1) that was dose-dependent but plateaued at maximum levels of inhibition with TGF-β (2 ng/ml). This dose response for inhibition of neutrophil attachment is consistent with reports for the effect of TGF-β on endothelial cell proliferation, locomotion, and angiogenesis (*8*). The response was blocked by a monospecific antibody against TGF-β (R&D Systems) and similar inhibition was demonstrated with recombinant (r) TGF-β (Genentech); the basal adherence of 0.149 ± 0.016 was reduced to 0.063 ± 0.002 by rTGF-β (2 ng/ml). This dose of TGF-β did not result in inhibition of endothelial cell proliferation; there was little or no change in the morphology or density of the endothelial monolayer as assessed by dye uptake and microscopy. Cell numbers after 24 hours of exposure to TGF-β (2 ng/ml) were $10.6 \pm 0.8 \times 10^3$ and without exposure to TGF-β were $9.6 \pm 0.8 \times 10^3$ (arithmetic mean ± SEM, $n = 5$) from an original 10^4 cells plated per well.

To test the effect of varying the time of addition of TGF-β on the inhibition of neutrophil attachment, endothelial cells were replated into microtiter wells, and treated for 3 to 24 hours with TGF-β (2 ng/ml) before assaying for neutrophil binding. Significant inhibition of adherence was seen after a 6-hour exposure; however, maximum inhibition was obtained when TGF-β was added at the initiation of the experiment and the endothelial cells were exposed for a full 24-hour period (Table 1). Again, no effect on endothelial cell growth was observed under these conditions.

TGF-β has been shown to decrease the number of high-affinity epidermal growth factor (EGF) receptors on a variety of endo-

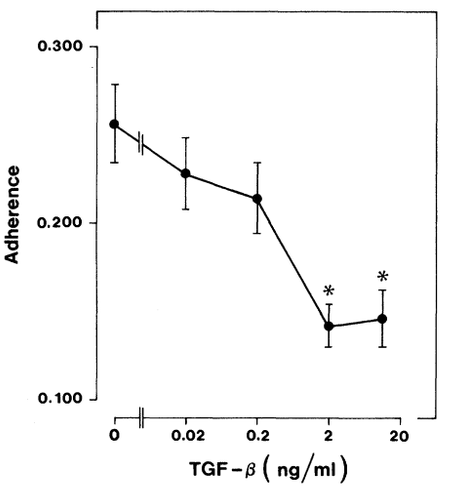

Fig. 1. The effect of TGF-β on adherence of neutrophils to endothelial monolayers. HUVEs obtained from collagenase treatment of umbilical cords were cultured for 3 to 7 days in 25 cm^2 flasks (Costar) in RPMI 1640 plus 20% fetal bovine serum (FBS) as described (*17*). Cells were then harvested by trypsinization (Flow) and plated into 6.41-mm^2 microtiter wells (NUNC) at 1.6×10^4 cells per well. TGF-β (97% pure isolate from human platelets, R&D Systems) was then added and after 24 hours the adherence of Ficoll-Hypaque–purified (*18*) venous blood neutrophils (mean purity 96%) was measured with the use of an assay based on the uptake of the viable stain Rose Bengal (*19*). Between 2 and 8×10^5 neutrophils in RPMI 1640 plus 5% FBS were added to wells of a 96-well flat-bottomed microtiter tray containing monolayers of HUVEs. After 30-min incubation at 37°C in 5% CO_2, the supernatant was removed and 100 μl of 0.25% Rose Bengal stain was added for 5 min at room temperature. Nonadherent cells were removed by two subsequent washes in medium; stain incorporated into the cells was released by the addition of 200 μl of ethanol:phosphate-buffered saline (1:1). After 45 min the wells were read in an enzyme-linked immunosorbent assay (ELISA) reader (Dynatech) at 570 nm. The level of adherence is given as the mean optical density reading at 570 nm (OD_{570}) of wells containing adhering cells and HUVEs minus the mean OD of wells containing HUVEs alone. On the average a tenfold change in OD_{570} represents a tenfold change in the numbers of cells adherent per square millimeter. Each point represents the arithmetic mean ± SEM of 9 to 15 determinations from four to five separate experiments. Points marked by an asterisk differ significantly from no added TGF-β ($P \leq 0.005$, two-tailed t test).

Division of Human Immunology, Institute of Medical and Veterinary Science, Frome Road, Adelaide, South Australia, 5000.