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-responsive Internal Ribosome Entry Site (IRES) Element in Human Methionine Synthase

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Regulation of homocysteine, a sulfur-containing amino acid that is a risk factor for cardiovascular diseases, is poorly understood. Methionine synthase (MS) is a key enzyme that clears intracellular homocysteine, and its activity is induced by its cofactor, vitamin B
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 does not bind to the MS initiation codon. Electrophoretic mobility shift analysis reveals the presence of a B
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-dependent protein-RNA complex and suggests the possibility that B
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-dependent increase of IRES efficiency is mediated via a protein. Modulation of the IRES-dependent translation of an essential gene by the cofactor of the encoded enzyme represents a novel example of a gene-nutrient interaction.

The important interplay between genes and nutrients modulates a number of physiological and pathophysiological processes (1). The influence of nutrients on DNA stability, repair, methylation, and gene expression is well studied (2). Elevated levels of homocysteine, a metabolic product of an essential nutrient, methionine, is correlated with an increased risk for cardiovascular diseases (3), neural tube defects (4), and Alzheimer’s disease (5). Intracellular clearance of homocysteine is controlled by the activity of three enzymes that are found at a vitamin-rich metabolic junction. These include the B
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-dependent cystathionine ß-synthase, B
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- and folate-dependent methionine synthase (MS) and betaine homocysteine methyl transferase. Of these, only MS is ubiquitous and remethylates homocysteine to methionine. Mutations in MS result in hereditary hyperhomocysteinemia (6, 7). The rich B-vitamin dependence of homocysteine metabolism has stimulated studies on the benefits of multivitamin treatment in lowering plasma homocysteine (8–11).

The activity of MS in cells cultured in normal medium is enhanced by supplementation with vitamin B
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, an observation that was first reported over 30 years ago (12). This regulation is exerted at the translational level. In this study, we demonstrate that translation of MS, which has a long and highly structured 5' untranslated region, is initiated from an internal ribosome entry site (IRES), which is modulated by B
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. The minimal IRES element spans 71 bases immediately upstream of the initiation codon. Electrophoretic mobility shift analysis reveals the presence of a B
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-dependent protein-RNA complex and suggests the possibility that B
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-dependent increase of IRES efficiency is mediated via a protein. Modulation of the IRES-dependent translation of an essential gene by the cofactor of the encoded enzyme represents a novel example of a gene-nutrient interaction.

EXPERIMENTAL PROCEDURES

Materials—Eagle’s MEM (minimum essential medium), OHCbl, AdoCbl, MeCbl, and CNCbl were purchased from Sigma. Fetal bovine serum was from HyClone. Cell lines were purchased from American Type Culture Collection. Radiolabeled [γ-32P]ATP (5000 mCi/mmol) was purchased from Amersham Biosciences. HeLa S100 cell extracts were purchased from Paragon.

Cell Culture Conditions—Cells were grown in Eagle’s MEM supplemented with 10% fetal bovine serum and incubated at 37 °C, 5% CO
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 derived from fetal bovine serum is present at a concentration of ~125 pm in this medium. For B
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 induction studies, the cells were grown to 60–80% confluency, and fresh medium supplemented with 5 mg liter
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 OHcbl (3.6 μM final concentration) was added. For reporter studies (luciferase and CAT), cells were grown in 6-well 35-mm plates, harvested, and lysed according to the manufacturer’s protocols (Promega).

Reporter Constructs—Plasmids pSVCAT/ICS/LUC, pSVCAT/BIP/LUC, and pSVhGAT/CAT/LUC were kindly provided by Dr. Maria Hatzoglou (Case Western Reserve University) and were initially developed in Dr. Peter Sarnow’s laboratory (Stanford University). The pSV-CAT/ICS/LUC vector contains 400 nucleotides of antisense antemapped cDNA of Drosophila melanogaster (17) in the intercistronic (ICS) region and was used as a negative control for IRES-mediated translation.
The plasmid pSVCAT/BIP/LUC contains the IRES of the immunoglobulin-binding protein (17) and was used as a positive control. The plasmid pSVhpCAT/BIP/LUC contains a stable hairpin inserted in front of the first cistron. The MS 5′-UTR (394 bases) was PCR-amplified and cloned into the Sall/NcoI sites of the pSVCAT/ICS/LUC plasmid by replacing the ICS sequence to give pSV CAT/MS1–394/LUC. Using a similar procedure, the vector pSVhpCAT/MS1–394/LUC was obtained. Deletion constructs containing the last 340, 270, 220, 140, and 71 bases of the MS 5′-UTR were generated by PCR and subcloned into the Sall/NcoI sites of the bicistronic vector (with or without the hairpin) as described above for the full-length construct. The deletion constructs containing the last 67, 64, 51, 52, 47, 42, 35, and 26 bases, respectively, of the MS 5′-UTR were generated by PCR and subcloned into the Sall/BstEII sites.

Plasmid MS-pGL3-basic was constructed by cloning the MS 5′-UTR into the HindIII/NcoI sites of the pG3L-basic-promoter-less vector (Promega). Mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene). The primers employed in this study are described in supplemental Table S1.

**Transient Transfection and Reporter Assays**—Transfections were performed using the Lipofectamine reagent (Invitrogen) for 293 cells or performed using the Lipofectamine reagent (Invitrogen) for 293 cells or with the Lipofectamine reagent (Invitrogen) for COS-1, HepG2, and NIH3T3 cells. Briefly, 1.5 μg of plasmid DNA was mixed with 10 μl of transfection reagent according to the manufacturer’s specifications, and the mixture was added to 6-well plates. When needed, B12 was added to half of the plates 24 h after transfection. At the end of the incubation, reporter gene activity was determined according to the vendor’s protocol (Promega).

**Bicistronic RNA Northern Analysis**—Analysis was performed as previously described (13). A 32P-labeled probe consisting of a DNA fragment encompassing 650 bases at the 5′-end of the luciferase gene was employed to detect the bicistronic message.

**In Vitro Transcription**—DNA primers containing a T7 promoter sequence were designed to amplify various fragments of the MS 5′-UTR. The PCR products were used as templates for in vitro transcription using the Ribomax kit (Promega) according to the vendor’s protocol. The transcripts were resolved on a 10% polyacrylamide gel, isolated and 5′-32P-labeled as previously described (18).

**In-line Probing of RNA Constructs**—Labeled RNA fragments were subjected to in-line probing as previously described (18). Briefly, ~1 nm 5′-32P-labeled RNA was incubated for ~40 h at 25 °C in 20 mM MgCl2, 50 mM Tris-HCl (pH 8.3), and 100 mM KCl in the presence or absence of ligand (i.e. 200 μM B12 derivative). The reaction mixtures were resolved on a 6–15% polyacrylamide gel (depending on the length of the RNA) and analyzed using a PhosphorImager.

**REMSAs**—For RNA electrophoretic mobility shift assays, DNA templates were generated by PCR using the following primers: Forward: TAATACGACTCACTATAGGGAGCCAGCCGAGGTCAAACG Reverse: GTTTTCACCAGCTCTTCCTTTCCT. The forward primer incorporated the T7 promoter sequence. RNA probes were synthesized by in vitro transcription with T7 polymerase (Maxiscript kit, Ambion) in the presence of [α-32P]UTP. 15,000 cpm of labeled RNA was incubated with 75 μg of HeLa S100 cell extract (Paragon) in a total volume of 15 μl at room temperature for 30 min, followed by the addition of 1 μl of RNase T1 (1 unit/μl) and incubated for an additional 10 min at room temperature. Heparin was added to a final concentration of 5 mg/ml. When indicated, different forms of cobalamin were added at a final concentration of 200 μM or cell extracts were pretreated with proteinase K (100 μg/ml) before mixing with the RNA. The ribonucleoprotein complexes were electrophoresed on a 4% native acrylamide gel and visualized by autoradiography.

**UV Cross-linking Experiments**—RNA-protein complexes for UV cross-linking were prepared as described above. Before adding RNase T1, the samples were transferred into a 96-well dish and irradiated on ice with a 254-nm UV light source at 400,000 μJ/cm2. RNA-protein complexes were then resolved by 12% SDS-PAGE and visualized by autoradiography.

**Statistical Analysis**—Each experiment was repeated at least three times. Statistical analysis was performed using one-way analysis of variance (Microcal Origin software), and results were considered significant if the p value was <0.05.

**RESULTS**

The MS 5′-UTR Contains an IRES Element—To determine whether the MS 5′-UTR is able to initiate translation internally, a series of bicistronic vectors were employed as shown in Fig. 1A. In these constructs, the first cistron (encoding CAT) is translated via a canonical cap-dependent mechanism. The second cistron (encoding LUC) is translated efficiently only if translation initiation occurs in the intercistronic region. The following vectors were tested: CAT/ICS/LUC as a negative control, CAT/BIP/LUC as a positive control (15), and CAT/MS 5′-UTR/LUC containing the MS 5′-UTR instead of the BIP sequence. To rule out the possibility that the observed IRES activity does not result from ribosomal read-through, vectors having stable hairpins preceding the first cistron were employed (Fig. 1A).

Translation of the first but not the second cistron (Fig. 1B, upper panel) is strongly inhibited by the presence of the hairpin in the CAT/MS 5′-UTR/LUC construct and in the positive control containing the BIP-IRES (Fig. 1B, lower panel). These results provide evidence that translation of the second cistron occurs by initiation in the intercistronic region and are consistent with the presence of an IRES element in the MS 5′-UTR.

As an additional control, the LUC/CAT activity was measured in 293 cells treated with rapamycin, which inhibits cap-dependent translation by promoting dephosphorylation and activation of 4E-BP1, a repressor of the cap-binding protein, 4E (19). A ~10-fold increase in LUC/CAT activity was seen in the presence of rapamycin and resulted from diminished CAT activity whereas the LUC activity was unaffected (data not shown).

A potential source of error in interpreting data for internal initiation of translation is the possible presence of a cryptic promoter in the test sequence, which would result in a monocistronic luciferase mRNA in addition to the bicistronic message. Cap-dependent translation of the monocistronic RNA would confound the results. We thus tested a luciferase reporter construct in which the SV40 promoter was replaced by the MS 5′-UTR. Only background levels of luciferase activity were detected demonstrating that the MS 5′-UTR does not exhibit promoter activity (Fig. 1C).

A second source of error that needs to be considered is the possible presence of a splice site in the MS 5′-UTR. In this case, even if a bicistronic message is produced initially, splicing could generate a smaller fragment containing the second cistron. However, Northern analysis revealed the presence of only a single message that is long enough to accommodate both cistrons, ruling out the presence of a cryptic splice site in the MS 5′-UTR (Fig. 1D).

Because optimal IRES-dependent initiation requires trans-acting factors that have a tissue-specific distribution (20), we have examined the efficiency of MS- and BIP-IRES activities in different cell lines (Fig. 1E). Efficiency of the IRES varied within a 2–5-fold range compared with the ICS-containing negative control. The pattern observed with the BIP-IRES was comparable to that reported previously in the same cell lines (20). It is interesting to note that the highest MS-IRES activity was
FIGURE 1. **MS translation is IRES-dependent.**

**A.** Bicistronic constructs used to probe IRES activity. The plasmid designated as MS and BIP have the full-length MS 5′-UTR or the BIP 5′-UTR inserted between the CAT and luciferase cistrons. hp refers to the hairpin present upstream of the CAT cistron. B, MS 5′-UTR displays IRES activity in a bicistronic test. The reporter activities for plasmids containing the MS 5′-UTR (upper panel) or the BIP 5′-UTR (lower panel), which served as a positive control, were measured as described under “Experimental Procedures.” C, the MS 5′-UTR does not harbor a cryptic promoter. Comparison of luciferase activity in a monocistronic reporter constructs containing an SV40 promoter or the MS 5′-UTR in place of the SV40 promoter. Luciferase levels are normalized to the CAT activity of a monocistronic plasmid that was used as a control for transfection efficiency as described under “Experimental Procedures.” D, the MS 5′-UTR does not harbor cryptic splice site. Northern blots of COS-1 cells that were transfected with the MS or BIP bicistronic plasmid constructs shown in A indicate the presence of intact bicistronic mRNA. E, MS-IRES efficiency in different cell lines. The MS and BIP bicistronic constructs were transfected into different cell lines. Activities are reported in comparison with the background ICS activity (negative control) that is set as 100% as described under “Experimental Procedures.”
observed in the 293 kidney cell line, in which MS activity is also reported to be highest (21).

**Deletion Mapping of the IRES Element**—To map the minimal sequence that is required for IRES activity, we generated a series of nested deletions from the 5′-end of the MS 5′-UTR and measured IRES activity in the resulting bicistronic constructs. Fig. 2 shows that the deletions modulate IRES activity, probably because of the inhibitory or stimulatory influences of different regions in the MS 5′-UTR. For instance, the first 54 bases of the MS 5′-UTR are inhibitory, and their deletion increases IRES activity. As the deletions progress toward the 3′-end, stimulatory regions are eliminated, as indicated by a decrease in IRES activity. However, the last 71 bases of the MS 5′-UTR retain ∼80% of the IRES activity versus the full-length MS 5′-UTR.

To confirm that the 71-mer indeed harbors IRES activity, we have tested a plasmid containing the 71 bases inserted in the intercistronic region and a hairpin preceding the first cistron. When the hairpin is present, translation of the first cistron is inhibited, whereas translation of the second cistron is enhanced, consistent with retention of IRES activity in this sequence (data not shown).

**MS IRES Is Modulated by B₁₂**—The boundaries of the MS-IRES element and of the B₁₂ responsive element reported earlier (13) overlap, and raises the obvious question as to whether or not the IRES activity is modulated by B₁₂. A B₁₂-dependent increase (∼60%) in the translational efficiency of the bicistronic vector was observed, and the effect was clearly specific to the second cistron, which is under control of the MS-IRES element (Fig. 3). The fold increase in translation in the reporter construct is lower than the B₁₂ effect on the endogenous gene (∼3.5-fold in COS-1 cells), which could result from placement of the MS 5′-UTR in an artificial context in the bicistronic vector. A similar observation has been reported for the X-linked inhibitor of apoptosis protein, which is elevated 3.5-fold upon radiation treatment but shows a 50% increase in reporter activity when its IRES element is inserted in a bicistronic vector (16, 22). B₁₂ had no effect on the LUC/CAT ratio in constructs containing the BIP IRES or the ICS sequence between the two reporter genes (not shown). It should be noted that the control cells are cultured in medium containing ∼125 pm B₁₂ as described under “Experimental Procedures.” Thus, the effect of B₁₂ supplementation, which activates MS (13), is correlated with IRES-dependent translation of MS in this study.

**Secondary Structure Probing of MS-IRES**—Computer modeling of the 71-mer using Mfold (24) predicts the presence of a hairpin (between bases −9 and −44) with a long stem (Fig. 4A). Interestingly, this particular hairpin is retained in secondary structure models for all the deletion constructs described in Fig. 2. To test whether this hairpin exists in vivo, we probed the RNA secondary structure using the in-line probing method (18, 25), which relies on degradation of RNA at room temperature and analysis of the resulting degradation pattern (Fig. 4B). Many of the regions that are predicted to be double-stranded in the stem (e.g. bases −36 to −44) are protected from degradation. In contrast, loops are more accessible to cleavage and generate strong banding patterns in the gel (e.g. bases −34, −26 and −25). We also used this method to determine whether B₁₂ binds directly to the MS 5′-UTR. This analysis is based on the premise that binding of a ligand to RNA can cause changes to the secondary/tertiary structure and thus influences the pattern of degradation (18).

Fig. 4B shows the degradation pattern of a transcript spanning the last 71 bases of the MS 5′-UTR in the presence or absence of B₁₂. None of the B₁₂ derivatives visibly altered the degradation pattern, which suggests but does not establish that B₁₂ does not bind directly to the MS 5′-UTR. We similarly found no evidence for modulation of degradation by B₁₂ of the full-length MS leader or of the last 140 bases of the leader (data not shown).

The degradation pattern is in generally good agreement with the predicted long hairpin secondary structure in the model for the 71-mer (Fig. 4). The stronger degradation bands are seen in the predicted loops whereas the protected regions correspond to bases in the stem. These results provide evidence for the in vivo existence of the predicted hairpin structure in the MS IRES-element.

**Fine Mapping of the Minimal IRES Element**—The 71-mer was employed further for fine mapping analysis to probe the essential features of a minimal IRES element. To this end, 3–5 bases were deleted consecutively, starting at the 5′-end of the 71-mer. The secondary structure model predicts that the bases −9 to −44 form a long hairpin (Fig. 4A) and its role in IRES activity is confirmed by the bicistronic reporter assays (see loss of activity in deletion constructs −42, −35, and −26 in Fig. 5). The integrity of the bicistronic message was established by Northern analysis (Fig. 5B). The equal intensity of miRNA in these sam-

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**FIGURE 2.** Deletion analysis of the MS-IRES. Progressive deletions were made from the 5′-end of MS 5′-UTR and the IRES activity was measured as described under “Experimental Procedures.” Activities are reported after subtraction of the background ICS activity (negative control) and compared relative to the −394 MS 5′-UTR containing construct, which was set at 100.

**FIGURE 3.** MS-IRES is modulated by B₁₂. Reporter activities were measured in COS-1 cells transfected with the MS bicistronic vector (containing 394 bases of the 5′-UTR) at the indicated times following treatment with B₁₂. (*) denotes a p value < 0.05.
ples is consistent with the reporter assays reflecting changes at the translational level.

The deletion analysis revealed that the sequence from −47 to −43 (CACGU) is important for IRES activity. In a Mfold analysis, both the last 47 and the last 42 bases of MS 5′-UTR form a stable hairpin with the only difference being that the main stem is 9-bp long in the 47-base hairpin and 7-bp long in the 42-base hairpin. To determine whether the primary sequence or the length of the stem was important, we mutated residues −44 and −43 and introduced complementary changes at −9 and −10 so that the 9-bp stem length was retained. The IRES activity of this mutant was 100% of the activity of the wild-type IRES (not shown). In contrast, mutating residues at the base of the stem so that pairing between residues −44 and −9 and −43 and −10 were disrupted resulted in −50% of wild-type IRES activity (not shown). This is consistent with the length of the hairpin stem being important rather than the specific sequence at the positions that were tested. However, these results do not exclude the possibility that the mutations disrupted a tertiary interaction that modulates IRES activity. Because of the small magnitude of the B12 effect in the bicistronic construct (Fig. 3), fine mapping of the B12 effect was not pursued in this study.

A B12-responsive Protein Binds to MS 5′-UTR—Whereas the structure probing data do not rule out direct B12 binding to the MS 5′-UTR (Fig. 4), they suggest that the B12 effect may be expressed via a trans factor. To test this hypothesis, we performed electrophoretic mobility shift assays to detect proteins that bind to the MS 5′-UTR. A labeled RNA probe encompassing the last 140 nucleotides of the MS 5′-UTR, which showed the highest B12-sensitivity in a reporter gene assay (13), was incubated with cytosolic S100 extracts from HeLa cells in the presence or absence of different forms of B12 and analyzed on native PAGE. Of the four complexes that were detected, three bind to the RNA independently of B12 (Fig. 6A). The fourth complex (with the highest molecular weight) exhibits B12 specificity, binding RNA in the order of decreasing strength in the presence of OHCbl>Mecbl>CNcbl. Binding was not observed in the presence of AdoCbl or in the absence of cobalamins. Preincubation of the cell extract with proteinase K prior to electrophoresis abolished complex formation confirming that binding of proteins to the RNA probe retarded its mobility (Fig. 6B). UV-cross-linking of the ribonucleoprotein complexes and separation on denaturing acrylamide gels provided an estimate of ~100 kDa for the molecular mass of the B12-responsive protein-RNA probe complex (data not shown).

DISCUSSION

Our studies reveal a novel mechanism of translational regulation of human MS that is IRES-dependent and is modulated by its cofactor, B12. The MS 5′-UTR is 394 bases long in contrast to the 5′-UTRs of most cellular mRNAs that are between 25 and 70 bases in length (26). The presence of extensive secondary structure (with a predicted ∆G of >−50 kcal/mol) in the 5′-leader sequence is generally believed to inhibit ribosome scanning (26). Secondary structure predictions for the full-length MS 5′-UTR, estimate a ∆G of −175 kcal/mol for melting the multiple hairpins. Structure probing experiments of the full-length MS 5′-UTR confirm that it is indeed highly structured raising the possibility that IRES-dependent initiation could be important for this leader-burdened mRNA.

Several cellular IRES elements are activated under conditions of stress (viz. amino acid starvation, irradiation, and apoptosis) during which general cap-dependent translation is inhibited (14–16, 27). IRES-dependent translation initiation confers an advantage under these conditions by bypassing general translation arrest and allowing expression of proteins essential for adaptation/survival. MS is an essential gene as revealed by the embryonic lethality of MS-null mice (28). IRES-dependent translation of MS could have evolved to maintain production of this enzyme under different environmental conditions. B12 is a relatively rare, albeit essential, vitamin with limited distribution and is absent in the plant kingdom. We have speculated previously that translational up-regulation of MS by B12 may represent an evolutionary adaptation to the presence of this nutrient (13), which leads to rapid synthesis and sequestration of the vitamin by the only known B12-utilizing enzyme in the cytoplasm, MS (29, 30).
FIGURE 5. Fine mapping of the MS-IRES. A, 3–5 nucleotides were consecutively deleted from the 5′-end of the 71-mer and analyzed in the bicistronic reporter assay as a ratio of LUC/CAT activity (solid bars). The deletions did not affect mRNA expression as indicated by the uniform expression of CAT activity in all the constructs (open bars). The activity for each construct is reported after subtraction of the background ICS activity (negative control) and compared with the −394 MS 5′-UTR construct, which was set at 100. B, Northern analysis demonstrates integrity of and equal expression of the bicistronic reporter mRNA in the deletion constructs. COS-1 cells were transfected with bicistronic plasmid DNA. After 24 h, the cells were harvested and subjected to Northern analysis using a luciferase probe as described under “Experimental Procedures.” The data are representative of three independent experiments.

FIGURE 6. RNA electrophoretic mobility shift analysis of RNA-protein complexes with the MS 5′-UTR. A, a B12-responsive complex and three B12-independent complexes form on the MS IRES element. Gel mobility shift assays were performed with a 32P-labeled probe encompassing the last 140 bases of the MS 5′-UTR and S100 extracts from HeLa cells as described under “Experimental Procedures.” B, the mobility shifted complexes disappear upon treatment with proteinase K (100 μg/ml (+) and 1 mg/ml (++) confirming the presence of protein-RNA complexes. AdoCbl, MeCbl, CN-, and OHCbl are cofactor derivatives that are photosensitive, and it is likely that the form of the vitamin that is delivered to the cytoplasm is predominantly in the MeCbl and AdoCbl states.

A B12-responsive IRES Element

directly to a responsive element in the MS 5′-UTR (Fig. 4). However, in combination with the observation that the MS 5′-UTR sequence is retarded by B12 only in the presence of cell extract (Fig. 6), they support mediation of the B12 effect by a protein factor.

IREs activities are modulated by a number of proteins named ITAFs (IRES trans-activating factors) such as the hnRNP I/polypyrimidine tract-binding protein (37), the La autoantigen (38) and the ribonucleoproteins C1 and C2 (39). Variation in the MS-IRES potency in different cell lines (Fig. 1E) suggests the involvement of tissue-specific expression of ITAFs interacting with the MS-IRES. Interestingly, factorless ribosome assembly on the IRES of cricket paralysis virus has been reported (40), and it was suggested that distinct pseudoknot-like structures are important for the correct positioning of the ribosome on the mRNA, bypassing the need for initiation factors.

Based on the current study, a model for regulation of MS translation initiation by its cofactor.

FIGURE 7. A B12-dependent protein factor, CarA, which is a transcriptional regulator in Myxococcus xanthus (42, 43). CarA is a repressor of the carB operon encoding carotenoid biosynthetic functions and has a DNA binding domain that is directly fused to a B12 binding domain. Binding of B12 to CarA is proposed to release the repressor from the promoter sequence. B12 has also been reported to inhibit the hepatitis C virus IRES-driven translation (44) by stalling the 80 S ribosomal complex on the IRES (23).
Our studies reveal the presence of three other protein complexes that bind to the MS 5′-UTR independently of B12 (Fig. 6). These putative ITAFs may be involved in modulating IRES activity. The identities of these proteins as well as of the B12 responsive protein are currently under investigation in our laboratory.

In summary, we have shown that MS, which is essential for survival, is translated via an IRES mechanism and that a protein factor appears to be involved in mediating the B12-dependent response of the IRES element. Modulation of IRES-dependent translation of MS by B12 represents a novel mode of gene regulation by a nutritional factor, which is also the cofactor for the encoded enzyme.

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