Control of MYEOV protein synthesis by upstream open reading frames
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Running Title: Control of myeov protein synthesis

The myeov gene has been isolated by the tumorigenicity assay and is localized at chromosome 11q13, a frequent site for chromosomal rearrangements in various carcinomas and B-cell neoplasms. In addition myeov is coamplified with cyclinD1 (CCND1) and overexpressed in carcinomas of various organs. The mechanisms of myeov regulation remain enigmatic. The 5’ untranslated region (5’-UTR) of the myeov gene is long, encompasses several upstream AUGs and is predicted to fold in a strong secondary structure suggesting that its translation might be regulated by an internal ribosomal entry site (IRES). Here we show that initial experiments using monocistronic and dicistronic reporter constructs supported this assumption. However the application of in vitro transcription/translation assays, Northern blot analysis and promoterless dicistronic constructs revealed promoter activity of the myeov 5’-UTR. DNA transfection of dicistronic DNA constructs, normal and mutated forms of myeov cDNA fragments cloned in a eukaryotic expression vector and direct RNA transfection analysis revealed that upstream AUG triplets in the 5’-UTR of the myeov transcript abrogate translation. Alternative splicing mechanisms in specific cell types and/or developmental stage may evade this translation control. Control experiments suggest that the 5’-UTR from encephalomyo-carditis virus (EMCV), when inserted at the midpoint of a dicistronic vector, is also able to function as a cryptic promoter.
cellular mRNAs including transcripts that mediate internal initiation during the inhibition of cap-dependent translation in mitosis and apoptosis, like fgf-2, pdgf-2, igf-II, vegf, odc, c-myc, xiap, apaf-1, bip (reviewed in Stonely and Willis (19); Holcik et al (20)). Many of the IRESs are GC-rich and contain complex secondary structures. However, no common structural features have been recognized to date.

Presently the concept of IRES-mediated translation initiation in eukaryotes is a topic of controversial discussion (21-23). The gold standard for the detection of internal ribosome entry site activity (IRES) is the dicistronic assay. In this assay the IRES is placed between two cistrons (chloramphenicol (CAT), Renilla/Firefly Luciferase (RLuc/FLuc) or other reporter genes) in a dicistronic vector and then transiently transfected into eukaryotic cells. Translation of the upstream ORF occurs via CAP-dependent scanning, whereas translation of the downstream cistron depends on the activity of the IRES. Low efficiency of translation and a lack of proper controls (e.g. Northern blotting) to exclude alternative splicing or the presence of a transcriptional promoter within the putative IRES may lead to a misinterpretation of the IRES activity for a given test sequence.

The 5' UTR of myeov possesses several features which superficially resemble mRNAs that purportedly translate via internal ribosome entry. Its 5' UTR has a length of 445 nucleotides, contains several upstream AUGs (uAUGs) that may generate four different polypeptides with a length of 22, 59, 11 and 7 amino acids and is predicted to fold in a complex secondary structure ($\Delta G = -153.1$ kcal/mole).

In this paper we describe the analysis of the 5’ UTR of the myeov gene. Initial data suggesting IRES activity were challenged by the application of in vitro translation and RNA transfection studies. In addition Northern blot analysis revealed the presence of a cryptic promoter. Finally we were able to show that the presence of the uAUG codons almost completely abrogates protein translation.

The myeov gene thus fits the paradigm wherein an encumbered 5’-UTR is used to limit translation of a potent protein that is likely to be harmful if overproduced (23).

### Experimental Procedures

#### Establishment of the mono- and dicistronic construct

- The luciferase plasmids: pGL3, pHNL, pRF, pHpRF and pRF+EMCV have been described previously and were kindly provided by Dr. A. Willis (24). Pfu DNA polymerase (Promega) was used to amplify the complete myeov 5’-UTR using a cloned cDNA 11SMNp14m81 encoding for the large ORF and the following oligonucleotides primers: myeovEcoRIUTRfor 5’- CGGAATTCCAGAACCACATCTCCCTACAAAAGC AG-3’ and myeov14NcoIUTRrev 5’- CATGCCATGGGCGAGGAGAGGAGGAGCCAG-3’. A’s were added by the addition of dATPs (Pharmacia) and Taq DNA polymerase and incubation at 72 °C for 30 minutes. The fragment was purified after gel electrophoresis using a gel extraction kit QIAEX II (Qiagen) and inserted into a T-vector, pGEM®-T Easy plasmid (Promega) originating pGEM-T+myeov 5’-UTR. The identity of the insert was verified by DNA sequencing using M13-forward and reverse primers. The fragment was digested by EcoRI and NcoI and cloned into pGL3, pHNL, pRF and pHpRF between the EcoRI and NcoI sites thus creating pGL3+5’-UTR, pHNL+5’-UTR, pRF+5’-UTR and pHpRF+5’-UTR. Again correct insertion was verified by the dideoxy terminator cycle sequencing method using a CycleReader™ Auto DNA Sequencing kit (MBI Fermentas) and a specifically labeled Firefly Luciferase reverse primer 5’-CTTCTGACCAACAGGAGCCGAGC-3’ on a Li-Cor 4200 DNA Analyzer (Li-Cor).

- Construction of the bicistronic promoterless constructs were performed by deleting the SV40 promoter sequence including the intron between the Smal and EcoRV sites from pRF, pHpRF+5’-UTR and pHpRF+EMCV by restriction digestion, agarose gel electrophoresis, purification by QIAEXII (Qiagen) and religation and thus creating pGL3(-P), pHNL(-P)+5’-UTR, pRF(-P)+5’-UTR and pHpRF+5’-UTR. Again correct insertion was verified by the dideoxy terminator cycle sequencing method using a CycleReader™ Auto DNA Sequencing kit (MBI Fermentas) and a specifically labeled Firefly Luciferase reverse primer 5’-CTTCTGACCAACAGGAGCCGAGC-3’ on a Li-Cor 4200 DNA Analyzer (Li-Cor).

- Construction of normal and 5’-UTR mutated myeov cDNA fragments into the eukaryotic expression vector pMTSM

- To analyze the effect of uORFs in the myeov 5’-UTR on protein translation, Pfu DNA polymerase (Promega) was used to amplify the complete myeov 5’-UTR using a cloned cDNA
11SMNp14m81 encoding for the large ORF and the following oligonucleotides:
meyeovUTR HindIII for 5'-
CAGCCCAAGCTT
CGGACCGCGAACCCACA TC
and myeyeovUTR EcoRV rev 5'-
GGTTCCGATATCGAGCCAGGGAAGGAGC C-3'. Fragments were treated with Taq polymerase and dATPs to add A's, resolved by gel electrophoresis, purified with QIAEX II (Qiagen) and cloned into pGEM®-T Easy vector, originating the construct pGEM-T-meyeov 5'-UTR HindIII/EcoRV. Inserts were verified by DNA sequencing. Single and combinations of mutations within the upstream AUGs of the meyeov 5’-UTR were introduced using pGEM-T-meyeov 5’-UTR HindIII/EcoRV as a template and the QuickChange® Multi Site-Directed Mutagenesis Kit as recommended by the manufacturer (Stratagene). The following oligonucleotides were used: meyeovATG 1 - 5’-
CAAGACAGGAAGTAAACCTGGAGGAGGC C-3’, meyeovATG 2 - 5’-
CAGAGGGCGGGAGAAGCCATCCCCACTG-3’, meyeovATG 3 - 5’-
GGGCCGGGGCGTGCAAGGCCTCAGGG-3’, meyeovATG 4 - 5’-
GGCCTCAGGGAAGGCCTGTTCAGCTGC-3’. Introduced mutations were verified by DNA sequencing and the inserts, meyeov 5’-UTRmut were recloned into pRF+EMCV upstream of the Renilla Luciferase cistron between the HindIII and EcoRV restriction sites. Correct insertion was verified by DNA sequencing using a specific labeled Renilla luciferase reverse primer 5’-
ACACCGCGCTACTG-3’.

To create constructs of meyeov cDNA fragments in the eukaryotic expression vector pMT2SM several amplifications were performed. The open reading frame without 5’- and 3’-UTR sequences and the ATG in an optimal Kozak context (pMT2SM+ORF(K)) was amplified using primers; meyeov-Kpn-Kozak-long 14: 5’-
GGGGTACC GCCACCATG GCCCTCAGAATCT GCG-3’ and meyeov Xba-end: 5’-
GCTCTAGATTCAACAAAGTGAGGATGATG GATGAT G-3’. The open reading frame without 5’- and 3’-UTR sequences and its own ATG (pMT2SM+ORF) was amplified using primers meyeov-Kpn-own-long 14: 5’-
GGGTTACCATCCCTCGGCTCAGATGGGCC and meyeov-Xba-end. Amplifications, cloning and verification were as described above. Fragments were cloned into pMT2SM using KpnI and XbaI. Complete meyeov cDNAs encoding the respective large (11SMNp14m82) and short ORF (11SMNp2m69) were digested with NotI from the lambda gt10 cloning vector and ligated into NotI digested pMT2SM. In addition following ligations were performed. The plasmid pMT2SM+5’-UTRmut+ORF+3’UTR was created by a double ligation. For that, the meyeov 5’-UTR containing the 4 mutations in the uORF was obtained from pGEM-T+UTR1,2,3,4mut and digested with NotI and PflII. The main ORF together with the 3’-UTR of meyeov was excised from the plasmid 11SMNp14m82 containing a complete meyeov cDNA encoding the largest ORF using the same restriction enzymes, polished with Pfu DNA polymerase and blunt-end ligated into the polished NotI site of pMT2SM. To generate a construct that lacks the 3’-UTR (pMT2SM+5’-UTR+ORF), the plasmid 11SMNp14m82 was digested with AatII, polished and subsequently digested with the restriction enzyme NotI. The fragment was applied to agarose gel electrophoresis, purified by QIAEX II (Qiagen) and ligated into the vector pMT2SM that was digested with EcoRI, polished and subsequently digested with NotI. To construct the plasmid lacking the 5’UTR (pMT2SM+ORF+3’-UTR) the plasmid 11SMNp14m82 was digested with PflII, polished and then digested with the restriction enzyme NotI. The fragment was purified by gel electrophoresis and QIAEX II and ligated into pMT2SM which was digested with PstI, polished and digested with NotI.

In vitro RNA synthesis and in vitro translation - pRF, pRF+5’-UTR and pRF+EMCV Luciferase fusion constructs were column-purified (Qiagen), and directly used to prime coupled in vitro transcription/translation reactions (TNT) according to the manufacturer’s instructions (Promega). 5 µl were withdrawn from this reaction in 10 min intervals, combined with an equal volume of 2 x Passive Lysis Buffer (Promega), and Renilla and Firefly Luciferase activity were determined as described below.

RNA transfection and quantitation of translation efficiency - For in vitro transcription experiments using T3 Polymerase, we used a bluescript based plasmid (Stratagene) containing the SHOX 5’-UTR upstream of the Firefly Luciferase cistron with the SV40 polyA
adenylation site and SV40 enhancer (25). In this case the SHOX 5'-UTR was replaced by either the wildtype or the mutated myeov 5'-UTR using the SpeI/NcoI restriction sites, originating pBSK+UTR and pBSK+UTRmut, respectively. Prior to in vitro transcription both constructs were linearized with the restriction enzyme XhoI. Synthetic mRNA was generated using the mMESSAGE mMACHINE™ T3 reaction system (Ambion) according to the manufacturer’s recommendations. The quality and the size of the mRNAs were analyzed by agarose gel electrophoresis and 2 µg of RNA were directly transfected into HEK 293 cells using 8 µl of TransMessenger™ Transfection Reagent (QIAGEN) per well of a 6-well plate according to the manufacturer’s protocol. Twenty-four hours after transfection, transfected cells were trypsinized, washed once with phosphate-buffered saline (PBS), and the cell pellet was resuspended in 1000 µl PBS. 200 µl were pelleted and resuspended into 100 µl 1 x Passive Lysis Buffer (Promega) and directly used for luciferase assays or first stored at – 70 °C. The remaining 800 µl were pelleted and used for poly A+ RNA isolation using the GenElute™ Direct mRNA miniprep kit of Sigma according to manufacturer’s recommendations.

Luciferase assay - For Luciferase measurement HEK-293 cells were lysed 24 to 48 hours after transfection with 1 x Passive Lysis Buffer (Promega) for 15 minutes at room temperature. 20 µl of each cell lysate were measured for Firefly and Renilla Luciferase activities using the Dual Luciferase Reporter System (Promega) and a dual injector 96-well plate luminometer (Anthos) as recommended by the manufacturer. All assays were performed at least three times and in triplicate.

Northern Blot analysis - Total cellular RNA and poly (A) were isolated with the High Pure RNA Isolation kit (Roche) and the GenElute™ Direct mRNA miniprep kit (Sigma) according to the manufacturer’s instructions. Northern Blot analysis and stripping were performed as described by Janssen et al., 1998 (27). Briefly, 10 µg of total RNA or 2 µg of poly A+ RNA were loaded onto a denaturing formaldehyde agarose gel, electrophoresed in the presence of formaldehyde and transferred to Nytran 13N nylon membranes (Schleicher and Schuell). Filters were hybridized in 3 x SSC (0.45 M NaCl, 0.045 M sodium citrate), 5 x Denhardt’s, 200 µg/ml denatured salmon sperm DNA, 1% SDS and 10% dextran sulphate at 63 °C for 16 hours with random primed labeled probes (MBI Fermentas). Filters were extensively washed in 3 x SSC, 0.1% SDS at 63°C, followed by a wash at higher stringency. Filters were exposed to Kodak Biomax MS film with a Kodak intensifier screen. The following probes were used for hybridization analysis: Firefly Luciferase probe generated by PCR using the following primers Lucy1for: 5’-GGAGAGCAACTGCATAAGGC-3’ and Lucy1rev: 5’-CATCGACTGAAATCCCTGGT-3’; Renilla Luciferase specific probe generated by PCR using the following primers; Ren for: 5’-ATGTTGTGCCACATATTGAGCCAGT-3’ and Ren rev: 5’-GATTTCACGAGGCCATGATAATGT-3’.

Cell culture and DNA transfection - HEK-293 cells were grown at 37°C in DMEM/HAM’S F-12 (1:1) (PAA Laboratories) supplemented with 10% FCS, 100 Units/ml penicillin, and 100 µg/ml streptomycin (PAA Laboratories) in a humidified atmosphere containing 5% CO2. One day before transfection 3 x 10^6 cells were seeded into 10 cm dishes. DMEM/HAM’S F12 medium was replaced with Dulbecco’s modified Eagle’s medium with supplements and DNAs were transfected by the calcium phosphate method (26). Two days after transfection cells were washed twice in PBS and 1/10 of the cells was lysed with 1x Passive Lysis Buffer (Promega) and used for Luciferase assays, 1/10 was used for Western Blot analysis and the remaining cells were used for RNA isolation. When transfections were performed with Gene Juice (Novagen), 100,000 cells/well were seeded into a 24 well plate one day before transfection. The next day 400 ng DNA and 8 µl Gene Juice reagent were used for transfection according to the manufacturer’s instructions.

S1 nuclease analysis - S1 analysis was performed using a fluorescently labeled ssDNA probe according to the protocol of Noti and Reinemann with some modifications (28). The regions spanning the 5'-UTR of the myeov and and IRES of the EMCV gene were first amplified by PCR using 25-ng of dsDNA of the pGL3-P+myeov 5'-UTR and pGL3-P+EMCV plasmids with 50 pmoles of primers in a standard 100 µl
Immunoblotting and antibodies - Proteins were resolved by 10% SDS-PAGE and blotted onto nitrocellulose (PROTRAN, Schleicher and Schuell Bioscience) by standard procedures. Filters were blocked with TBST (10 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) +10% carnation for 1 hour at room temperature. MYEOV antibodies were added at 1 µg/ml in 10 ml TBST+10% carnation and incubated overnight at 4°C. Following incubation with goat-anti-rabbit antibodies (1:10,000) filters were developed with ECL reagent (Amersham/Pharmacia). MYEOV polyclonal antibodies were obtained by immunizing rabbits with a MYEOV-specific peptide corresponding to amino acids 103-116 (NH2-CAGDRERNKGDKGAQ) and affinity purified on a peptide column containing immobilized MYEOV-specific peptide (FZB Biotechnik GmbH, Berlin).

RESULTS

Structural features and predicted secondary structure of the myeov 5'-UTR. The myeov 5'-UTR has a length of 445 nucleotides and contains four AUG codons (uAUGs) upstream of the main open reading frame (ORF). These AUGs are associated with ORFs of 22, 59, 11 and 7 amino acids, respectively. Considering the importance of nucleotides at position -3(A/G) and +4(G) in the Kozak’s consensus sequence for optimal translation (A/G)CC(A/G)CCAUGG) (29), the first three AUGs show only one of the two optimal features, while the fourth AUG shows both features, albeit followed by a stop codon (Fig. 1). Structure-prediction algorithms using the mfold 3.1 algorithm (30,31) predicts a strong secondary structure of this 5'-UTR with a Gibbs free energy $\Delta G = -153.1$ kcal/mole for the most stable configuration. The presence of uAUGs and stable secondary structure should cause ribosome scanning to be inefficient. This suggests that myeov mRNA translation might be regulated by a cap-independent mechanism (e.g., internal ribosome entry).

Effect of the myeov 5'UTR on translation of downstream open reading frames. In order to evaluate its effect on translation, we cloned the myeov 5'-UTR upstream of a Firefly Luciferase (FL) reporter gene into the monocistronic plasmid pGL3 using the myeov reaction during 40 cycles, respectively. For myeov we used the following primers; pGL3RVprimer 3' as a forward primer: 5'-ctagcaaaataggctgcccagt-3' and a fluorescently labeled MyeovPromPErev2 primer: 5' DY-681-ggccctgaggtcaggtcaggg-3'. For EMCV we used the identical forward primer and a fluorescently labeled EMCVrev2 primer: 5'-DY681-gttgccctgagcccagaaag-3'. Approximately 100 ng of ds template and 20 pmols of labeled primer were then subjected to 30 cycles of asymmetric PCR in a standard 100-µl reaction volume. The labeled ss PCR product (along with the ds template) was electrophoresed in one lane of a 2% low melting point (LMP) minigel. The ssDNA, which runs at approximately one-half the molecular weight of the ds template, was visualized on a UV transilluminator, excised from the gel and trimmed of excessagar with a scalpel. The agarose was melted at 65 °C and an equal volume of 10T0.1E buffer (10mM Tris-Cl, 0.1 mM EDTA, pH 8.0) was added. RNA was isolated from HEK 293 cells that had been transfected using Gene Juice (Novagen) and the plasmids pGL3-P+myeov 5'-UTR and pGL3-P+EMCV. Poly A' RNA was isolated using the Gene Elute Direct mRNA MiniPrep Kit (Sigma) according to the manufacturer’s instructions. For S1 analysis, 2 µg of poly A' RNA were ethanol-precipitated and resuspended in DEPC-treated H2O, dried and redissolved in 20 µl S1 hybridization buffer (80% deionized formamide, 0.4 M NaCl, 1 mM EDTA, 50 mM piperazine-N,N', -bis(2-ethane-sulfonic acid)(PIPES), pH 6.4). Approximately 3 µl of preheated (65 °C) of ss probe DNA was added, heated at 80 °C for 5 minutes and hybridized overnight at 30 °C. After hybridization 119 µl of DEPC-treated H2O, 15 µl 10 x S1 buffer (300mM sodium-acetate (pH 4.6); 10 mM Zinc acetate, 50% glycerol), 15 µl 3M NaCl and 1 µl of S1 nuclease (25 Units) (Invitrogen) were added and incubated for 60 min at 25 °C. The reaction was stopped with the addition of 10 µl 0.5 M EDTA, 40 µl 7.5 M ammonium acetate, 5 µg yeast tRNA and 1 ml of ethanol. Following ethanol precipitation, the pellet was resuspended in 2 µl of DEPC-treated H2O and 1 µl of Sequence loading buffer was added and analyzed on a Li-Cor 4200 DNA Analyzer (Li-Cor) together with a sequence reaction of the plasmid DNAs, obtained with the same primer used to prepare the ss probes.
AUG (pGL3+UTR) (Fig. 2). Human embryo kidney 293 cells were transfected with pGL3 and pGL3+UTR, and the FL activity from each construct was determined (Fig. 2). The FL levels were corrected for transfection efficiency using Northern blot analysis and Phosphor Imager quantitation. The presence of the myeov 5'-UTR upstream of the FL reporter gene reduced its activity by ~ 98 % indicating that this sequence modulates gene expression (Fig. 2). In addition, we wanted to determine whether MYEOV protein synthesis can proceed in a cap-independent manner, i.e. by IRES. For this reason an inverted repeat sequence forming a very stable hairpin structure (-55 kcal/mole) was placed downstream of the SV40 promoter, creating phpL and phpL+UTR (Fig. 2). As a consequence ribosome scanning from the 5' CAP site is severely impaired while ribosomes entering distal of the hairpin will be unaffected. HEK-293 cells transfected with this construct almost completely abolished (~98 %) cap-dependent translation (Fig. 2). However, introduction of the myeov 5'-UTR sequence between the hairpin and the reporter gene stimulated Luciferase activity ~ 6-fold above that observed with phpL (Fig. 2). These data suggest that the myeov 5'-UTR can direct translation in a cap-independent manner. A similar effect has been described for many other genes containing IRES sequences and indicates the presence of an IRES within this leader sequence.

**Analysis of IRES activity in the myeov 5'-UTR using the bicistronic vector.** To verify whether the myeov 5'UTR contains an IRES we inserted it into the bicistronic vector pRF between the Renilla (RL) and Firefly Luciferase (FL) reporter genes (32). This plasmid contains two reporter genes, the first cistron (Renilla Luciferase) is under the control of a SV40 promoter and translated via a cap dependent mechanism, while the second cistron (Firefly Luciferase) is translated independently of the cap structure, e.g. by internal ribosome entry. The myeov 5'UTR was cloned upstream of the Firefly Luciferase cistron originating pRF+UTR (Fig. 3). These plasmids were transiently transfected into 293 cells and the activity of both Luciferases was determined 48 hours after transfection. The activity of the Firefly Luciferase (second cistron) was normalized to that of Renilla Luciferase (first cistron) to correct variations in transfection efficiency. Figure 3b shows that the Firefly Luciferase is increased 9-fold over background after insertion of the myeov 5'-UTR into the intercistronic position. Different control assays were performed to check whether alternative mechanisms such as enhanced ribosomal reinitiation at the Firefly Luciferase initiation codon and/or the generation of Firefly Luciferase mRNAs due to alternative splicing, or the presence of a cryptic promoter might be responsible for this result.

To demonstrate that the observed IRES activity is not due to ribosome reinitiation, we used the phpRF vector. This vector contains an inverted repeat sequence upstream of the RL coding region, which produces a stable hairpin structure in the mRNA (-55 Kcal/mole). CAP-dependent translation of the upstream RL cistron should be greatly diminished, whereas cap-independent IRES activity of the downstream FL cistron should not be affected (Fig. 3). As expected, the hairpin structure reduced RL expression in both constructs, phpRF and phpRF+UTR (data not shown). In addition, uncorrected background FL activity was also reduced in phpRF compared to pRF (data not shown). The uncorrected FL activity of phpRF+myeov was only slightly reduced compared to pRF+myeov (data not shown). Compared to the empty construct phpRF, insertion of the myeov 5'-UTR induced FL activity by ~ 16-fold (Fig. 3). This suggests that translation initiation driven by the myeov 5'-UTR is not dependent on ribosome scanning from the 5' end of the dicistronic RNA and therefore cannot be due to ribosome reinitiation.

In addition to the SV40 promoter, the dicistronic vector pRF also contains a T7 promoter situated downstream of the SV40 promoter. This T7 promoter is used when pRF constructs are tested in an *in vitro* RNA synthesis and Translation system using T7 polymerase for transcription of the RNA and a nuclease-treated rabbit reticulocyte lysates to translate the transcribed RNA. pRF, pRF+myeov 5'-UTR and pRF+EMCV 5'-UTR were tested in this *in vitro* assay (Fig. 4). In this assay translation of Renilla Luciferase (RL) also occurs via cap-dependent scanning, whereas translation of Firefly Luciferase depends on the presence of an IRES in the intercistronic region. Although pRF+EMCV
showed ~ 10-fold induction of corrected FL activity, pRF+myeov did not show any induction (Fig. 4). We reasoned that a lack of specific IRES transactivation factors (ITAFs) in the rabbit reticulocyte lysate necessary for IRES-dependent translation may explain our failure to demonstrate IRES activity in the myeov 5'-UTR (24,33).

To ensure that the observed IRES activity in the myeov 5'-UTR is not due to the presence of cryptic promoters or splice sites in the 5'-UTR, we deleted the SV40 promoter sequences in the dicistronic constructs, creating pRF(-P), pRF(-P)+myeov 5'-UTR and pRF(-P)+EMCV 5'-UTR (Fig. 5). In this situation, no RNA transcription can occur and as a consequence no translation should follow. Surprisingly pRF(-P)+myeov as well as pRF(-P)+EMCV exhibited ~ 10-fold higher FL activity than the empty construct pRF(-P), suggesting that a cryptic promoter is present in the myeov- and EMCV 5'-UTR (Fig. 5).

Northern blot analysis demonstrates promoter activity in the myeov 5'-UTR. Despite the fact that different experiments using monocistronic and dicistronic constructs containing the myeov 5'-UTR indicated the presence of an IRES sequence, we also observed some discrepancies. Our in vitro experiments using a combined transcription/translation system did not support the idea of IRES and our control experiments with promoterless constructs showed unexpected activity. In addition recent publications insist that RNA analysis should be performed to uncover alternative transcripts originating from alternative splicing and/or promoter activity when using dicistronic constructs and this information prompted us to perform a Northern blot analysis. Despite low FL activity in our last experiments employing the promoterless constructs, Northern blot analysis revealed a clear FL-transcript in cells transfected with pRF(-P)+ myeov 5'-UTR (Fig. 5, insert). Unexpectedly cells transfected with pRF(-P)+EMCV 5'-UTR also showed a weak FL transcript (Fig. 5, insert). These data indicate the presence of a promoter sequence in the 5'-UTR of myeov and EMCV. Northern blot analyses of other transfection experiments using pRF+myeov 5'-UTR and phpRF+myeov 5'-UTR constructs confirmed these data and revealed two transcripts originating from the SV40 promoter and the cryptic promoter in the 5'-UTR (Fig 3b, inserts).

The intensity of the two transcripts is almost the same and indicates that the cryptic promoter exhibits rather strong activity. Northern blot analyses of cells transfected with a promoterless monocistronic construct (pGL3) containing the myeov 5'-UTR confirmed these data and analyses of dicistronic constructs containing different deletions in the myeov 5'-UTR mapped this promoter activity between the second and third uAUG (data not shown).

Mapping the mRNA initiation sites of the cryptic promoters in the myeov 5'-UTR and EMCV IRES. In order to localize the mRNA initiation sites of the cryptic promoter sites in the myeov-5'-UTR and the EMCV IRES sequence we performed S1 analysis. SsDNA probes were generated from the corresponding regions and hybridized to RNA of HEK 293 cells transfected with the pGL3-P+myeov 5'-UTR and pGL3-P+EMCV, that both showed cryptic promoter activity by Northern blot analysis. S1 analysis mapped several initiation sites of the myeov cryptic promoter in the 5' end of its 5'-UTR (Figs. 1 and 6). The relative position of the initiation sites corresponds with our deletion mapping data (see above). As the cryptic promoter activity of the EMCV IRES was very weak (Fig. 5, insert) we were only able to map 3 weak mRNA initiation sites in the 5' end of the EMCV IRES (Fig. 6). The presence of additional weak mRNA initiation sites can not be excluded.

Upstream ORFs inhibit translation of the downstream MYEOV ORF. Despite strong activity of the cryptic promoter in the myeov 5'-UTR, the observed FL activity of pRF+myeov 5'-UTR is rather low compared to the activity of the upstream RL cistron (1%). These data suggest that, although there is strong FL transcription from the cryptic promoter in the myeov 5'-UTR, translation of this transcript is severely impaired. In order to evaluate the effect of the various uORFs present in the myeov 5'-UTR on translation of the main ORF, we performed several experiments. In the first experiment we used pRF+EMCV 5'-UTR whereby the EMCV-IRES driven cap-independent FL translation was used as an internal control for transfection efficiency. The myeov 5'-UTR was inserted upstream of the RL cistron. In addition we analyzed a construct containing the myeov 5'-UTR devoid of any
uAUGs (Fig. 7). The corrected FL activity was high in the empty vector, returned to basic levels when the myeov 5'-UTR was inserted upstream of the RL cistron and was almost completely restored after removal of all uAUGs (Fig. 7). These results show that upstream AUG triplets in the 5'-UTR of the myeov transcript control protein synthesis.

The contributions of the four individual uAUGs to the repression of translation were evaluated using constructs containing combinations of various AUG→AAG mutations. Different mutations and different combination of mutations abolished translation, but a clear preference could not be observed (data not shown).

In order to directly analyze the effect of uAUGs in the myeov 5'-UTR RNA without the interference of possible transcriptional effects due to, for example, promoter competition for transcription factors, we transiently transfected in vitro transcribed RNAs directly into HEK-293 cells and measured FL activity. For this experiment we used a bluestrip-based vector containing the T3 promoter followed by the FL cistron (Fig. 8). The myeov 5'-UTR and a mutated form in which all uAUGs were altered, was placed upstream of the FL cistron. Plasmids were linearized, transcribed in vitro using T3 polymerase, analyzed on a gel, blotted, hybridized with an FL probe and quantitated by a Phosphor-Imager. Equimolar amounts of RNA were transiently transfected into HEK-293 cells and FL activity was measured. Insertion of the myeov 5'-UTR drastically abolished the translation efficiency of the FL cistron, while mutation of the uAUGs restored FL activity to its normal level (Fig. 8).

Finally we cloned the complete myeov cDNA sequence, a cDNA sequence in which all uAUG in the 5'-UTR were mutated and cDNA sequences that were missing either the 5'-, the 3'-UTR or both, into the eukaryotic expression vector pMT2SM (Fig. 9). Constructs were transiently transfected into HEK 293 cells and after 48 hours RNA and protein were isolated. The respective RNAs were analyzed by Northern blotting using a myeov 5’ probe and proteins were analyzed by Western blotting using MYEOV specific antibodies (Fig. 9). Northern blot analysis showed specific transcripts of expected sizes. In the first lane we show the analysis of a transfection in which the start codon of the main myeov ORF was altered into an optimal Kozak sequence, cloned into pMT2SM and transiently transfected into HEK-293 cells (Fig. 9). Only one MYEOV-specific protein band can be seen. Transfection of the normal myeov ORF with its own AUG revealed two bands in the Western blot that most likely represent the normal ORF of 313 amino acid residues and a protein product that starts at the second AUG in the same open reading frame resulting in a protein with a length of 255 amino acid residues (lane 2). Despite strong RNA expression of the two complete myeov cDNA constructs containing the short and long ORF respectively, no protein product could be detected (lanes 3 and 4). Mutation of the uAUGs in the myeov 5'-UTR restored protein translation (lane 5). Deletion of the myeov 3'-UTR had no effect (lane 6), but again deletion of the 5'-UTR containing the 5'-uAUGs restored translation of the MYEOV ORF, although RNA expression level was rather low (lane 7). The last two experiments again show that upstream AUG triplets in the 5'-UTR of the myeov transcript can regulate the expression on the translation level.

DISCUSSION

We have characterized the 5'-UTR region of the myeov gene using several techniques. Its relatively large size of 445 bp, the fact that it can form a very stable secondary structure and the presence of several uAUG with uORFs suggested that translation of the myeov gene might be regulated by an IRES sequence. Some viral and cellular RNAs use IRESes to initiate translation in a cap-independent manner. The RNA of these viruses is naturally uncapped and utilizes the IRES sequence to recruit ribosomes to the vicinity of the initiation codon and facilitate translation. Under conditions of cellular stress when cap-dependent translation is severely impaired, cellular IRESes still allow translation of the mRNAs and escape control mechanisms of cap-dependent translation (34). Most cellular IRESes have been identified in the mRNAs of genes encoding oncoproteins, growth factors and proteins regulating apoptosis.

Insertion of the myeov 5'-UTR upstream of the Firefly Luciferase cistron in a monocistronic reporter vector dramatically reduced translation efficiency. Placing a stable hairpin downstream of
the promoter sequence greatly reduced Firefly Luciferase activity. However, insertion of the 
myeov 5'-UTR stimulated Luciferase activity approximately 7-fold. These data suggest that 
myeov translation may be initiated by an alternative mechanism, e.g. internal ribosome 
entry. To substantiate this hypothesis we inserted the 
myeov 5'-UTR into the dicistronic construct 
RF to give pRF+myeov 5'-UTR. Introduction of the 
myeov 5'-UTR stimulated Firefly Luciferase activity 9-fold. This number is comparable to the 
data published for most of the other putative IRESes present in cellular mRNAs (reviewed in 
Kozak et al. 2001 (21)).

To demonstrate that this result is due to 
the presence of an IRES and not a consequence of ribosomal readthrough from the first cistron, a 
hairpin was inserted upstream of the Renilla 
Luciferase to create the plasmids phpRF and 
phpRF+myeov 5'-UTR. Renilla activity dropped 
~95%, indicating that the hairpin efficiently abrogated ribosomal scanning. Again, introduction of the 
myeov 5'-UTR stimulated Firefly activity almost 16-fold, suggesting that this effect is not 
due to ribosomal readthrough.

In most previous studies the dicistronic 
construct has been used to prove the presence of an IRES in a 5'-UTR. However its use is heavily 
debated since it has been recognized that the presence of a cryptic promoter and/or potential 
splices site may lead to misinterpretation of the results (21-23,34,35). We therefore performed 
several control experiments, namely 1) in vitro 
transcription and translation of the dicistronic construct harboring the myeov 5'-UTR, 2) usage of 
a promoterless dicistronic vector and 3) Northern 
blot analyses of RNA from the transfected cells.

Our in vitro transcription/translation data 
showed induction of Firefly activation in the control construct pRF+EMCV. In contrast 
pRF+myeov 5'-UTR did not show activation when compared to empty vector. Although these data 
argue against the presence of an IRES, this result can be justified by explaining that cell type 
specific IRES trans-acting factors (ITAFs) were missing in this experimental setting.

The application of promoterless constructs 
should rule out the existence of cryptic promoters in the cellular IRES. In this vector the unique 
SV40 promoter together with the intron from the dicistronic constructs is simply removed. When 
transfected into 293 cells, no transcription will 
occur and therefore no translation (Firefly activity) 
should be measured. Except for the empty vector, 
pRF(-P)+myeov 5'-UTR as well as pRF(-P)+EMCV showed Firefly Luciferase activity, 
implying that the myeov 5'-UTR may enclose 
the presence of promoter sequences and therefore not an IRES sequence. This was confirmed by Northern blot 
analyses showing a smaller Firefly transcript 
originating from the myeov 5'-UTR. These data 
support other publications which warn that 
additional methods should be applied, such as the 
usage of promoter-less constructs, Northern 
blotting, RT-PCR, RNAi-based methods and RNA 
transfections, when using the dicistronic system. 
(21,22,34,35). It is also noticeable that we detected 
a faint but clear transcript in RNA of 293 cells 
transfected with a dicistronic construct harboring the EMCV IRES in the intercistronic region. It is 
generally accepted that this sequence is supposed 
to contain an IRES sequence. The reason why the 
insertion of the EMCV IRES in the midpoint of a 
dicistronic vector is able to function as a cryptic 
IRES is enigmatic. Retrospectively we 
analyzed RNAs from 293 cells transfected with empty pRF or phpRF vector and pRF+myeov 5'- 
UTR and phpRF+myeov 5'-UTR construct DNAs. 
Only dicistronic constructs containing the myeov 
5'-UTR in the intercistronic region showed an 
additional smaller transcript. The fact that this 
transcript did not hybridize to a Renilla Luciferase 
probe and can also been seen when using 
promoterless constructs implies that it originates 
from the myeov 5'-UTR. Albeit the presence of a 
IRES was identified by Northern 
analyses of cells transfected with mono- and 
dicistronic constructs, sequence analyses of 
several 5' RACE cDNA fragments as well as 
database analysis of human EST clones, did not 
reveal the presence of shorter myeov transcripts 
(data not shown). These data suggest that in its 
normal genomic context this promoter is either not 
used at all or perhaps only at a specific 
developmental stage, in a specific cell type or 
under distinct conditions (stress, apoptosis, oxygen 
depletion, etc.).

Experiments using dicistronic constructs, 
mRNA transfection and transfection of mutated 
and nonmutated fragments of the myeov cDNA 
demonstrated that translation of myeov is 
attenuated by the presence of uAUGs. Single and
combined mutation analyses of the uAUGs revealed that all four uAUGs had a moderate effect on translation. Attenuation of translational efficiency by the presence of uORFs is well known for genes encoding proteins controlling cell growth and differentiation, whose translation efficiency is tightly regulated (11,36). An example is the proinsulin gene, where low levels of proinsulin biosynthesis in the embryo result from the presence of uAUGs within a specific form of embryonic proinsulin mRNA. (37). A 32 nucleotides extended leader region containing two uAUGs in the embryonic mRNA abrogates proinsulin biosynthesis. Similar mechanisms may also prevent harmful overproduction of the MYEOV protein. The biological role of MYEOV has not been elucidated yet, but regulation of transcription, translation and posttranslational modifications may depend on the developmental stage or tissue distribution, as described for the proinsulin gene (37). Indeed, compared to other tissues pancreas tissue shows anomalous myeov RNA species. These pancreas specific mRNAs may differ in their 5’-UTR sequences and regulate MYEOV biosynthesis in a tissue specific manner (8).

A recent test of putative IRESes in the 5’-UTR of bad, sno, hiap and eIF4 applying a new promoterless construct unexpectedly revealed strong promoter activity (35). Our study is in line with these data and stresses that it is of utmost importance to examine more rigorously the claim that there is IRES activity in cellular mRNAs. In addition, we showed that translation of MYEOV protein is tightly regulated by uAUGs in the 5’-UTR. This regulation of MYEOV biosynthesis and its biological function deserves further attention.

REFERENCES

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FIGURE LEGENDS.

Fig. 1. Nucleotide sequence of the myeov 5′-UTR. The sequence of the 5′ region of the myeov gene from the transcription initiation site to the start codon. Upstream AUGs are underlined and numbered. Translation of the MYEOV protein starts at the fifth AUG (AUG 5). The deduced amino acid sequence is shown in the one-letter code. Messenger RNA initiation sites of the cryptic promoter are marked by arrow heads above the sequence. The position of the primer used to prepare the ssDNA probe and for sequencing is marked by an arrow below the sequence.

Fig. 2. Myeov 5′-UTR reduces translation efficiency of reporter Firefly Luciferase activity and a hairpin structure at the 5′ end of monocistronic mRNA does not block translation via myeov sequences. (a) The monocistronic Luciferase reporter vector pGL3 (1). The myeov 5′-UTR was inserted at the restriction sites indicated to create the construct pGL3+UTR (2). To construct phpL (3), a stable hairpin (-55 kcal/mol) that blocks ribosomal scanning was inserted downstream of the SV40 promoter into the vector pGL3. The myeov 5′-UTR was inserted at the indicated restriction sites to create the construct phpL+UTR (4). (b) HEK-293 cells were transfected with each of all four different constructs. The Luciferase activities were measured and normalized to Firefly Luciferase mRNA levels obtained by Northern blot analysis and phosphorimager quantification. The inserts show a representative Northern blot analyses of RNA from transfected cells hybridized with a Firefly Luciferase specific probe.

Fig. 3. Myeov 5′-UTR supports translation of the 3′ cistron in a dicistronic vector and a stable hairpin appended to 5′end of dicistronic mRNA does not block translation. (a) The dicistronic expression cassette of the vector pRF (1). Putative myeov IRES sequences are inserted in the intercistronic region, creating
Fig. 4. Effect of the myeov 5'-UTR on reporter gene expression in vitro. (a) Schematic diagram of the dual-Luciferase dicistronic constructs, pRF, pRF+myeov 5'-UTR (pRF+UTR) and pRF+EMCV 5'-UTR (pRF+EMCV). The constructs pRF+UTR and pRF+EMCV were generated by inserting the myeov and EMCV 5'-UTR in the intercistronic region. (b) The respective constructs were transcribed and translated in vitro using T7 polymerase for transcription of the RNA and a nuclease-treated rabbit reticulocyte lysates to translate the transcribed RNA. Renilla and Firefly Luciferase activities were determined after 80 minutes incubation. Firefly Luciferase activities were normalized to Renilla Luciferase levels.

Fig. 5. Cryptic promoter activity of the myeov 5'-UTR. (a) Schematic diagram of the promoterless dicistronic constructs pRF-P, pRF-P+UTR and pRF-P+EMCV. The sequences of the promoter and chimeric intron were removed from the parental dicistronic constructs shown in Fig. 4. (b) The promoterless constructs were transfected in conjunction with pCMV-LacZ into HEK-293 cells, and 48 h following the transfection, cells were harvested for determination of Renilla (Rluc) and Firefly Luciferase (Fluc) and β-galactosidase activities. Firefly and Renilla Luciferase activities were normalized to β-galactosidase activity. The relative values of Firefly Luciferase activities were calculated and normalized to that of the empty vector (pRF-P), where pRF-P was set to 1. The insert shows a representative Northern blot analysis of RNA from transfected cells hybridized with a Firefly Luciferase specific probe.

Fig. 6. Mapping of the cryptic promoter activity in the myeov 5'-UTR and the EMCV IRES by S1 analysis. (a) The S1 analysis of the myeov 5'-UTR is depicted in the lane marked S1. S1 products are marked by arrows. Poly A+ RNA of HEK 293 cells transfected with the pGL3-P+myeov 5'-UTR construct was hybridized with a fluorescently labeled ss DNA probe, treated with S1 nuclease and run on a sequence gel. The sequence of the myeov 5'-UTR and the position of the primer used to prepare the ssDNA probe and for sequencing are shown in fig.1. (b) The S1 analysis of the EMCV IRES is depicted in the lane marked S1. S1 products are marked by arrows. Poly A+ RNA of HEK 293 cells transfected with the pGL3-P+EMCV construct was hybridized with a fluorescently labeled ss DNA probe, treated with S1 nuclease and run on a sequence gel. The sequence of the EMCV IRES, obtained with the same primer used to prepare the ss probe is shown. (c) Nucleotide sequence of the EMCV IRES. Messenger RNA initiation sites of the cryptic promoter are marked by arrow heads above the sequence. The position of the primer used to prepare the ssDNA probe and for sequencing is marked by an arrow below the sequence.

Fig. 7. Effect of the various uORFs present in the myeov 5'-UTR on translation of the 5' ORF. (a) Schematic diagram of the dicistronic constructs pRF+EMCV, pRF+EMCV+UTR w/o mutations and pRF+EMCV+UTR with mutations. In the latter construct all four upstream AUGs of the myeov 5'-UTR are replaced by AAG. (b) The various dicistronic constructs were transfected into HEK-293 cells, and 48 h following transfection, cells were harvested for determination of Firefly and Renilla Luciferase activities. In this situation Renilla Luciferase activities were normalized to Firefly Luciferase levels. The actual measured activities were as follows (mean(SD)): for pRF+EMCV, Fluc 2035(480), Rluc
76406(5182); for pRF+EMCV+5’UTR without mutations, Fluc 1286(448), Rluc 2361(215); for pRF+EMCV+5’UTR with mutations, Fluc 431(129), Rluc 12359(1000).

**Fig. 8.** Effect of the various uORFs present in the *myeov* 5’-UTR on translation of the reporter gene by RNA transfection. (a) Schematic diagram of the constructs pBSK, pBSK+UTR and pBSK+UTRmutated. The constructs pBSK+UTR and pBSK+UTRmutated were generated by inserting the *myeov* 5’-UTR and a mutated form of the *myeov* 5’-UTR in which all 4 uAUGs were mutated upstream of the Firefly Luciferase cistron. (b) Equal amounts of *in vitro* transcribed RNAs from the T3 promoter were transfected directly into HEK 293 cells and FL activity was measured. Firefly Luciferase activities were normalized to Firefly Luciferase mRNA levels obtained by Northern blot analysis and Phosphor Imager quantification. The insert shows a representative Northern blot analysis of RNA from transfected cells hybridized with a Firefly Luciferase specific probe.

**Fig. 9.** Effect of various uORFs present in *myeov* 5’-UTR on translation of MYEOV protein. (a) Schematic diagram of the different *myeov* constructs in the eukaryotic expression vector pMT2SM. Different combinations of fragments or mutated fragments of the complete *myeov* cDNA were inserted behind the adenovirus promoter of the pMT2SM vector. Filled and open arrowheads indicate the positions of a translation initiation site in a perfect Kozak context or in its own suboptimal Kozak context, respectively. The shorter open reading frame is derived from a differently spliced *myeov* cDNA clone. (b) Different cDNA constructs were transfected into HEK-293 cells and 48 h following the transfection, cells were harvested for RNA and protein extraction. RNAs were analyzed by Northern blot analysis (N.B) using a specific 5’ *myeov* (position 1-894) probe and proteins were analyzed by Western blotting (W.B) using MYEOV-specific antibody. Positions of MYEOV proteins derived from the short (S) and long (L) ORF.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 7.
Figure 8.
Figure 9.
Control of MYEOV protein synthesis by upstream open reading frames
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