**Dissertation**

**submitted to the**

**Combined Faculties for the Natural Sciences and for Mathematics**

**Of the Ruperto-Carola University of Heidelberg, Germany**

**for the degree of**

**Doctor of Natural Sciences**

**presented by**

**BSc and MSc in Biomedicine and Biotechnology**

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**born in São Paulo, Brazil**

**Thesis Defence: January 24th, 2005**

# Molecular characterization of the putative oncogene *myeov*

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The research described in this thesis was carried out in the Institute of Human Genetics of the Medical Faculty of the Ruprecht-Karls-University of Heidelberg under the supervision of PD. Dr. J.W.G. Janssen and Prof. Dr. C.R. Bartram.

This work was financially supported by a grant from the "Deutsche Krebshilfe" to PD Dr. J.W.G. Janssen.

**To my parents**

# 1. Introduction

## 1.1. The *myeov* gene

For several years our group, using the tumorigenicity assay (section 1.2) and DNA from a gastric carcinoma, detected a potential oncogene. The DNA from a tertiary nude mice tumor (section 1.2) was cloned into EMBL-3 phage and screened with a human specific repetitive Alu-probe. Alu-positive phage clones were isolated and submitted to exon-trap analysis (Auch and Reth, 1990). Isolated exon fragments were used to screen a cDNA library from RNA of a tertiary nude mice tumor and a novel putative oncogene, designated *myeov* (myeloma overexpressed gene), was isolated.

Further analysis of this gene by fiber FISH-analysis, using the cell line (KMS-12) isolated from a patient suffering from a multiple myeloma (MM) with the t(11;14)(q13;q32), enabled the localization of this gene to chromosome band 11q13, 360-kb centromeric of the *cyclin D1* oncogene (Janssen et al., 2000). All breakpoints in mantle cell lymphomas (de Boer et al., 1995; Vaandrager et al., 1997a; Vaandrager et al., 1996) and MM cell lines (Gabrea et al., 1999; Raynaud et al., 1993; Ronchetti et al., 1999; Vaandrager et al., 1997b) occur in this 360-kb region between the *cyclin D1* and *myeov* genes. In addition, three out of seven MM cell lines carrying the t(11;14)(q13;32) showed overexpression of *myeov* on the mRNA level. *Cyclin D1* was overexpressed in all of these cell lines. Mapping analysis showed, that *myeov* and *cyclin* D1 came under the separate control of two different IgH enhancers, i.e. 3`E-α and 5`Eμ, respectively (Janssen et al., 2000). A similar activation mechanism has also been described for Follicular lymphoma (common type of non-Hodgkins`s lymphoma) exhibiting the reciprocal t(14;18)(q13;q21), in which the anti-apoptotic *BCL2* gene on chromosome 18 is juxtaposed to the IgH-Eμ enhancer on chromosome 14, and activated (Hockenbery et al., 1990).

The 11q13 region is involved in genetic rearrangements in a variety of human malignancies, including reciprocal translocations in B-cell neoplasms, unbalanced translocations or chromosomal inversions and frequent DNA

amplification in various carcinomas (Callanan et al., 1996; de Boer et al., 1997; Gaudray et al., 1992). *Cyclin D1* (CCND1) seemed to be a major candidate gene and has been described to be involved in B-cell lymphomas, breast tumors, and head and neck cancers (Callender et al., 1994; Dickson et al., 1995; Schuuring, 1995; Vaandrager et al., 1996). In breast cancer, amplification of the 11q13 locus has been correlated with a poor prognosis. The amplification is linked to lymph node metastasis and reduced survival (Cuny et al., 2000; Schuuring et al., 1992).

Amplification at the chromosomal region 11q13 is also observed in esophageal squamous cell carcinomas (ESC) and many others types of solid tumors (Schuuring, 1995; Schwab, 1998; Yoshida et al., 1993). This amplification is suggested to be linked to the malignant phenotypes of ESC, such as invasiveness, metastasis, and poor prognosis (Adelaide et al., 1995; Shinozaki et al., 1996; Yoshida et al., 1993). Within the 11q13 amplicon, *CCDN1* and *EMS1* were the only genes known to be amplified and overexpressed; therefore these genes were the major candidate genes in tumors comprising an 11q13 amplification (Hui et al., 1997; Schuuring, 1995). As we localized the *myeov* gene in this same amplicon, our group investigated the possible involvement of *myeov* in ESC carcinogenesis, and found that the *myeov* was coamplified together with *CCND1* in a great number of cell lines and primary tumors tested. However, *myeov* RNA overexpression was only detected in a subset of cell lines carrying *myeov* amplification. Aberrant methylation of the *myeov* promoter is responsible for this effect. Treatment of the cells with the demethylating agent 5-aza-2`-deoxycytidine restored *myeov* expression (Janssen et al., 2002).

Zoo blot analysis of the *myeov* gene revealed that the *myeov* gene is present in monkeys and humans, but is not conserved in fish, frog, sheep, mice and rats. Northern blot analysis showed that *myeov* is poorly expressed in most human tissues. Interestingly *myeov* is overexpressed in pancreas tissue, and shows anomalous *myeov* transcripts.

Transient expression of a GFP-MYEOV construct into Hela cells revealed expression in the endoplasmatic reticulum and in mitochondria. After

removing all of the *myeov* leucine-isoleucin tail, MYEOV proteins were localized in the mitochondria (unpublished data).

Although we detected the human *myeov* gene in the DNA of tertiary tumors (section 1.2) we were never able to transform NIH/3T3 cells using *myeov* cDNA. A possibility that another human oncogene is present in these tertiary tumors therefore still existis. The complete cloning and sequencing of the human DNA present in these tertiary tumors (generally ~40 kb) should reveal whether this is the case. A computer search with the deduced MYEOV protein sequence did not detect any homology with known protein motifs or domains. Its possible function is therefore still enigmatic.

## 1.2. Tumorigenicity assay

The NIH/3T3 focus assay (Shih et al., 1981) is a method to identify novel potential oncogenes. The method is based on the introduction of DNA from human tumors or cell lines into mouse fibroblastic cells NIH/3T3 by the calcium phosphate precipitation method (Graham and Eb, 1973) and screening for morphologically altered cells. Unfortunately there are human oncogenes which fail to promote changes in cells morphology. Because of this limitation, an *in vivo* assay variation of this analysis was created, named tumorigenicity assay (Brown et al., 1984; Fasano et al., 1984). In this assay, human tumor DNA is cotransfected together with plasmid DNA containing the neomycin selection marker into NIH/3T3 cells. Cells resistant to G418 are isolated and introduced subcutaneously into nude mice. The presence of an activated oncogene in the DNA may lead to tumor induction (primary tumor) in these nude mice. DNA from this primary tumor, containing the human activated oncogene, is isolated and the same transfection procedure is repeated again. A secondary tumor, now containing a small amount of human DNA encompassing the activated human oncogene is expected. A third transfection cycle may further purify the human oncogenic sequence, ending up with a tertiary tumor in which the human oncogene is the only human DNA present in these cells (Figure 1.1).

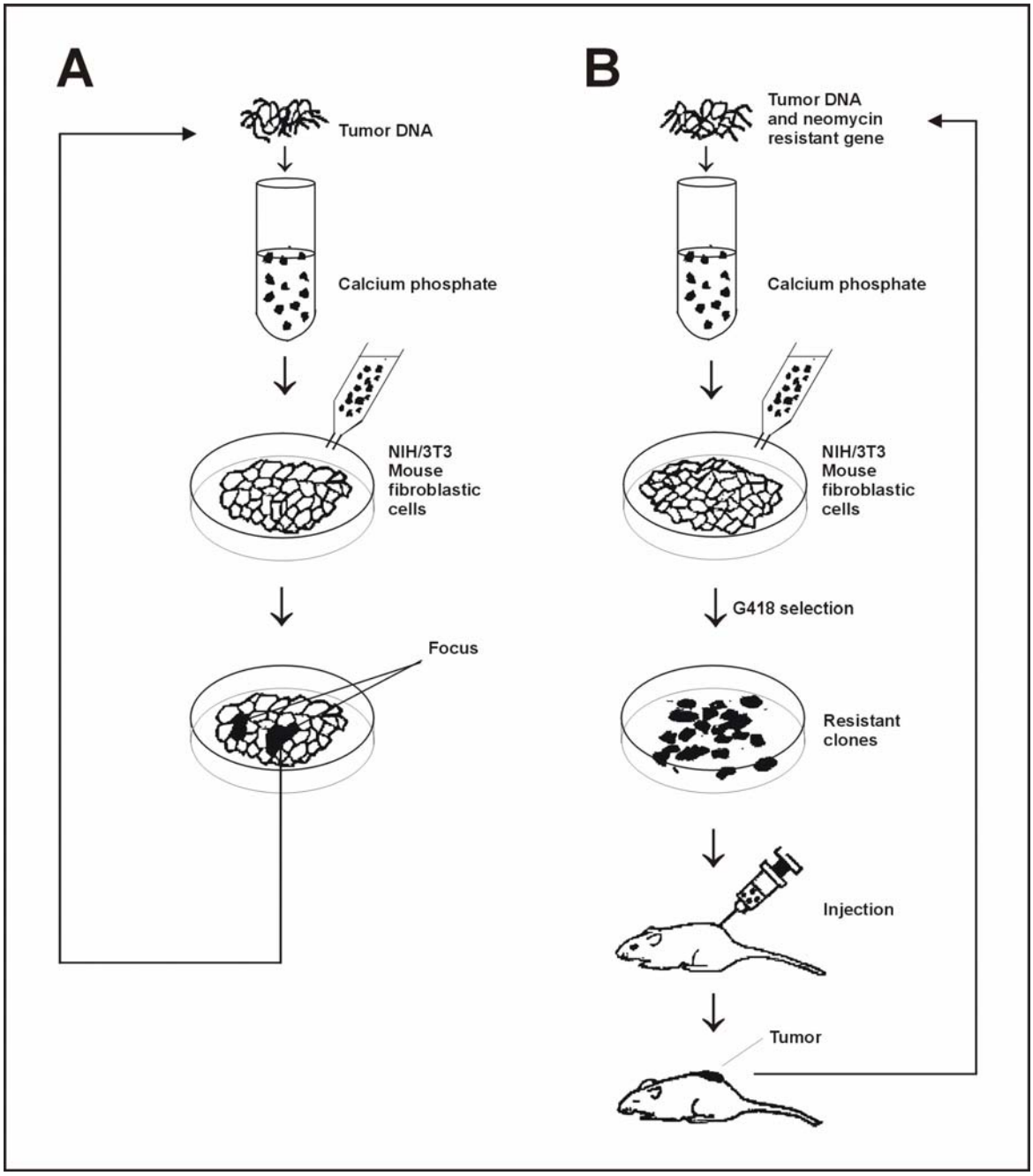



**Figure 1.1. Two methods for identification of oncogenes.**
(A) The classical NIH/3T3 focus assay. Human tumor DNA is isolated and transfected into NIH/3T3 mouse fibroblastic cells by the calcium phosphate precipitation method. The cell which takes up an activated human oncogene, present in the human DNA, may change its morphology and will grow out to a focus of morphologically transformed cells. In this case, the focus of transformed cells is isolated and DNA is extracted and the cycle is repeated once more. (B) Tumorigenicity assay. Human tumor DNA together with plasmid DNA encoding a neomycin resistant gene are cotransfected into NIH/3T3 mouse fibroblastic cells by the calcium phosphate precipitation method. G418 resistant cells are subsequently introduced into nude mice by subcutaneous injection. In case an activated human oncogene is present in the transfected DNA, this will lead to tumor formation. Human activated oncogenic sequences can be further purified by repeating this procedure. Finally we end up with a secondary or tertiary mouse tumor in which the human activated oncogene is the only human DNA present in these cells.

DNA of this tertiary tumor can be digested with a restriction endonuclease, and the resulting restriction fragments are cloned into phage DNA, packaged, incubated with bacterial cells and plated on agar plate. Replica filters of the resulting phage plaques are hybridized with a $^{32}$P-labelled human repetitive Alu-fragment. Alu-repeat sequences are human specific, are present in high copy numbers (~600,000 copies dispersed in the human genome) and are not present in the mouse genome. Human alu-repeat positive plaques are picked, phage DNA is isolated and human genomic DNA inserts are further characterized by sequencing. The classical NIH/3T3 assay and its variants allowed our group and others to detect several oncogenes (Table 1.1).

**Table 1.1. Detection of activated genes using the tumorigenicity assay**

| **Origin** | **Transforming gene** |
|---|---|
| monocytic cell line | *Mas* |
| myeloproliferative disorder | *Ufo* |
| myeloproliferative disorder | *p85β/HUMORF8* |
| monocytic cell line | *Ret* |
| erythroblastic cell line | *Cot* |
| monocytic cell line | *Tre* |
| Neuroblastoma | *FGF4 (hst)* |
| chronic myelocytic leukemia | *Dbl* |
| Neuroblastoma | *B-raf* |
| stomach carcinoma | *Myeov* |
| Monocytic cell line | *Gef-h1* |

(Brecht et al., 2004; Janssen et al., 1999)

## 1.3. Gene expression

DNA is packed into chromatin, a nucleoprotein complex that includes both histone and non-histone proteins. Chromatin organization within chromosomes is not uniform, with two distinct regions: euchromatin and

heterochromatin. Heterochromatin domains are in general inaccessible to DNA binding factors and are transcriptionally silent. Euchromatic domains in turn are actively transcribed (Grewal and Moazed, 2003).

The expression of genetic information in all cells is very largely a one-way system: DNA → RNA → Protein. DNA specifies the synthesis of RNA and then RNA specifies the synthesis of polypeptides, which subsequently form proteins. In order to express a gene, two successive steps are essential in all cellular organisms: transcription and translation (Strachan and Read, 2004).

### 1.3.1. Transcription

In the nucleus of eukaryotic cells DNA sequences are transcribed into RNA, in a complex mechanism called transcription. Although the transcription machinery of eukaryotes is much more complex than that of prokaryotes or archaea, the general principles of transcription and its regulation are conserved (Hahn, 2004). Transcription of eukaryotic and prokaryotic genes requires promoters and protein factors for transcription initiation (Landick, 2001; Nickels and Hochschild, 2004; Tariverdian and Buselmaier, 2004). The key enzymes in this process are RNA polymerases. Bacteria and archaea have only one polymerease that is responsible for transcription (Hahn, 2004; Nickels and Hochschild, 2004). In contrast, the nucleus of eukaryotes contains three different RNA polymerases (Table 1.2)(Hahn, 2004; Zorio and Bentley, 2004).

**Table 1.2. Eukaryotic RNA polymerases**

| Type | Location | Cellular transcripts |
|---|---|---|
| I | Nucleolus | 18S, 5.8S, and 25-28S rRNA |
| II | Nucleoplasm | mRNA precussors and snRNA |
| III | Nucleoplasm | tRNA and 5S rRNA |

All these polymerases recognize distinct promoters. Promoters contain a group of short sequence elements that are clustered, upstream of the coding sequence of a gene. Promoters and other cis-acting elements are binding

sites for subunits of the transcriptional machinery (Hahn, 2004; Tariverdian and Buselmaier, 2004). The binding of a transcription factor promotes the recognition of the transcription start site by RNA polymerase, and in turn results in transcription initiation (Zorio and Bentley, 2004).

Besides promoters, there are other cis-acting elements that regulate transcription. These sequences are called operators and enhancers, and they are found in prokaryotes and eukaryotes, respectively. Operator sequences are recognized by repressor proteins which inhibit transcription that would otherwise occur from the promoters, and enhancer sequences are positive control elements recognized by activator proteins that stimulate transcription from the promoter. In addition, silencer sequences have been found in promoter regions; where binding of proteins to the sequences negatively influence gene expression (Smale and Kadonaga, 2003; Struhl, 1999; Tariverdian and Buselmaier, 2004).

The effect of an enhancer or silencer can be blocked by the presence of an insulator sequence. In addition, insulators are able to establish independent domains of transcriptional activity within eukaryotic genomes (Fourel et al., 2004; Kuhn and Geyer, 2003).

The first complementary copy of the DNA is called primary transcript or pre-mRNA. Primary transcripts suffer several modifications. The major changes in the mRNA are capping, splicing, and poly A site cleavage (McCracken et al., 1997a; McCracken et al., 1997b) which are briefly described below.

### 1.3.1.1. mRNA processing

When nascent RNA is about 22 – 40 bases long, the phosphate group of the triphosphate at the 5`end is hydrolyzed and the remaining diphosphate group attacks to the α-phosphorus atom of GTP to form a 5`-5`triphosphate linkage. This special unusual linkage is called cap structure (Chiu et al., 2002; Moteki and Price, 2002; Rasmussen and Lis, 1993; Tariverdian and Buselmaier, 2004). The cap is methylated by action of a methyltransferase, which adds a methyl group to the N7 position of GMP cap to form the transcript's mature

$m^7G(5`)ppp(5`)N$ cap structure (Hirose and Manley, 2000). All transcripts derived from polymerase II are marked at their 5`ends with this cap structure (Zorio and Bentley, 2004), which protects the mRNA 5`end from exonucleases, facilitates splicing and transport to the cytoplasm, and supports binding of the 40S subunit of ribosomes to the mRNA (Mamane et al., 2004; Moteki and Price, 2002; Muthukrishnan et al., 1978; von der Haar et al., 2004).

The second important modification of the pre-mRNA is splicing. Splicing is a process which removes the introns (sequences that usually do not code for protein sequence), and joins together the rest of the sequence (exons). As a consequence mature mRNA only contains exon sequences. The coding region (from the start to the stop codon) is called open reading frame, the rest of the exon sequences are defined 5`- and 3`UTR (Tariverdian and Buselmaier, 2004). Exons can be joined to each other in different combinations, which provide one single gene the opportunity to express several different transcripts, coding for different proteins.

Splicing is mediated by a large RNA-protein complex, called spliceosome (Jurica and Moore, 2003; Tariverdian and Buselmaier, 2004). RNA splicing requires the nucleotide sequences at the exon/intron boundaries (splice junctions) to be recognized. In most of the cases, introns start with GU and end with AG (Strachan and Read, 2004; Tariverdian and Buselmaier, 2004).

Most mRNAs are processed at their 3`ends by cleavage and polyadenylation. A specific endonuclease recognizes the sequence AAUAAA and G/U rich sequence elements and cleaves the primary RNA at that site. Subsequently, a poly(A) polymerase adds 200 to 300 adenylate residues to the 3`end of the primary transcript (Barabino and Keller, 1999; Hirose and Manley, 2000; Tariverdian and Buselmaier, 2004). This terminus is called poly(A) tail, and is important for mRNA stability, transport of the mRNA to the cytoplasm and proper translation of the mRNA (Jacobson, 1996; Kaufmann et al., 2004).

## 1.3.2. Protein Synthesis

Protein translation is the term given to a process where the information of the nucleotide sequences of the mRNA is translated into amino acid sequences. This special and key process takes place in the cells cytoplasm, and proceeds on ribosomes, where transfer RNA (tRNA) and diverse proteins cooperate to produce the building blocks of life – the proteins.

Protein synthesis in eukaryotes resembles the one found in prokaryotes, however, there are basic factors that will be discussed below that differ between these two systems. In general, protein synthesis in eukaryotes is more complex.

In eukaryotes the start codon is generally an AUG (a few exceptions have been described where, ACG, CUG or GUG are used instead), albeit the methionine utilized differs from the one used in prokaryotes, since this one is not formylmethylated, and is called Met-tRNA$_i$. In contrast to prokaryotic, eukaryotic mRNA is usually monocistronic. However, many plants and animal viruses produce dicistronic or polycistronic mRNAs, but still only the 5`cistron is translated. To enable viruses to translate these 3`cistrons, additional forms of mRNA are produced in which the downstream cistron is repositioned closer to the 5`end via splicing (Kozak, 2002b).

In eukaryotes no Shine-Dalgarno like sequences have been identified, but eukaryotic mRNAs posses a cap structure at their 5`end, which is extremely important for initiation of ribosome scanning, and it will be discussed later in further detail.

Eukaryotic ribosomes are larger than prokaryotic ribosomes, exhibiting a mass of 4200 kDa. They also consist of two subunits: one large (60S) and one smaller (40S). The large subunit contains three RNAs: 5S, 25-28S and 5.8S (Granneman and Baserga, 2004; Nissan et al., 2002). The first two are counterparts of the prokaryotic ribosomal RNAs 5S and 23S, and the last one (5.8S) is exclusively present in eukaryotic ribosomes. The small subunit contains an 18S RNA that is homologous to the prokaryotic 16S RNA. There are three binding sites for tRNA on the ribosome: an A site, to which aminoacyl tRNAs are delivered in an mRNA-directed fashion, a P site where petidyl tRNAs

reside, and an E site through which deacylated tRNAs pass as they are released from the ribosome (Moore and Steitz, 2003; Roll-Mecak et al., 2001). For a more extensive description of the ribosome structure and its biogenesis see: (Dahlberg, 2001; Granneman and Baserga, 2004; Moore and Steitz, 2003; Nissan et al., 2002).

In contrast to prokaryotes where translation initiates immediately after transcription, eukaryotic mRNAs are first processed and specific modifications are made (section 1.3.1.1) before the mRNA can be transported to the cell's cytoplasm, where ribosomes will have access to the mRNA in order to start protein synthesis.

The most important checkpoint in protein translation is considered to be the initiation step, which is facilitated by proteins called eukaryotic initiation factors (eIFs). In eukaryotic system, there are more than ten eIFs involved in translation initiation (Preiss and Hentze, 2003). The factors that are involved in translation initiation are known and they are used to describe the model of protein translation called cap-dependent translation (Merrick and Hershey, 1996). According to this model (Figure 1.2), the 5`cap structure attracts the initiation factor eIF4E, which in turn recruits the cap-binding complex eIF4F. This complex consists of the DEAD-box RNA helicase eIF4A, eIF4G and even eIF4E itself. The factor eIF4E binds to the N terminus of eIF4G, which acts as an adaptor because it contains also a binding sites for eIF4A, poly(A) binding protein (PABP) and eIF3. The factor eIF4A, together with the RNA-binding protein eIF4B or eIF4H (not shown in the Figure), is believed to melt the RNA secondary structures near to its 5`end (Dever, 2002; Svitkin et al., 2001). In summary, binding of the cap-structure to the eIF4F complex recruits the 43S complex.

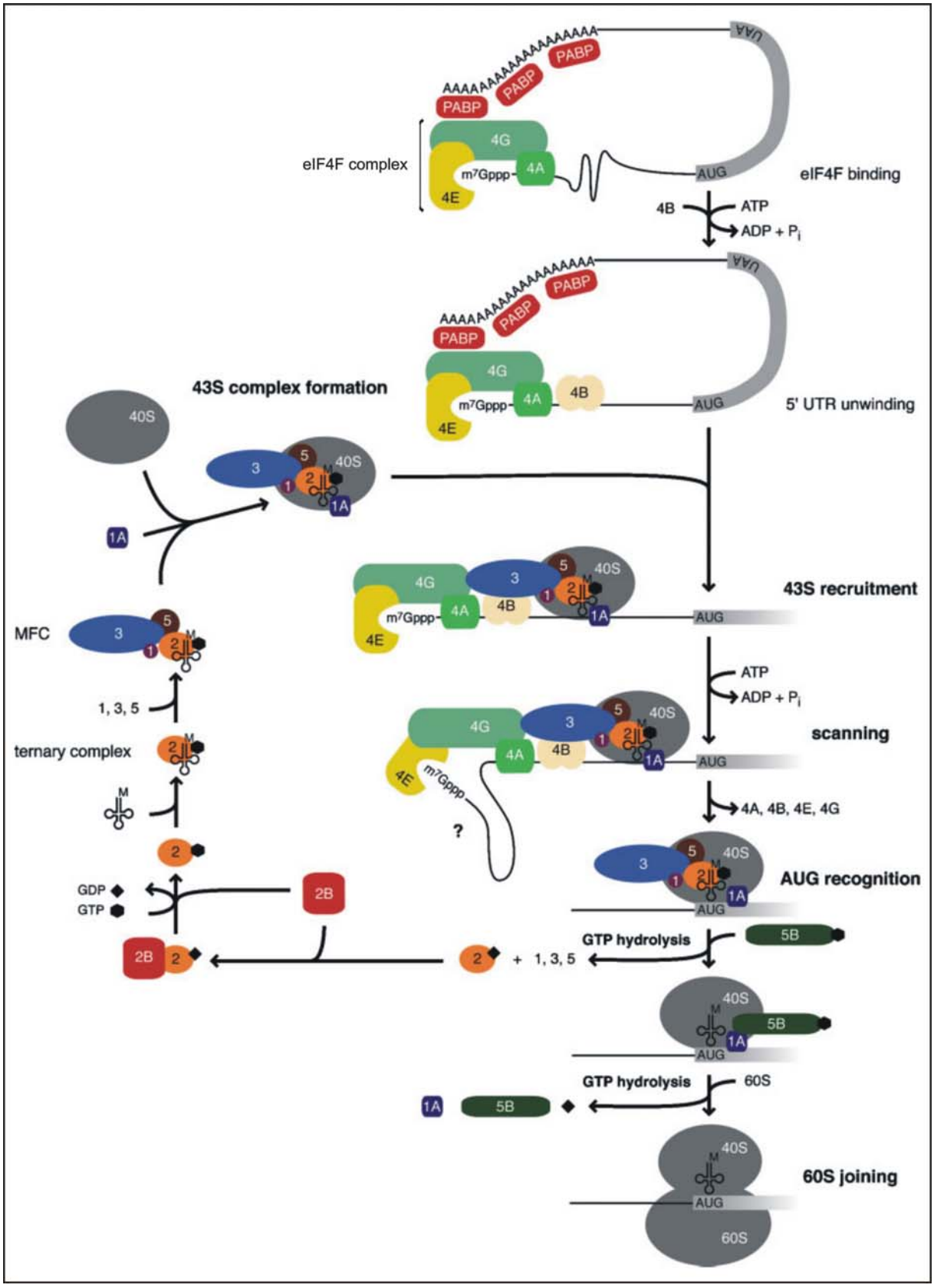



**Figure 1.2. The translation initiation pathway (Preiss and Hentze, 2003)**

The eIF2 has the important responsibility to bind to Met-tRNA$_i$. Since the GDP-bound form of eIF2 generated by each initiation cycle cannot bind MetRNA$_i$, it requires the action of the guanine nucleotide exchange factor (GEF) eIF2B. The eIF2 with GTP bind to MetRNA$_i$ creating a ternary complex (Preiss and Hentze, 2003). The factors eIF1, eIF3 and eIF5 interact with the ternary complex forming a so called in yeast multi factor complex (MFC). Binding of the ternary complex to the 40S ribosomal subunit is aided by eIF1, eIF1A, and the multisubunit factor eIF3, creating a 43S complex (Dever, 2002; Preiss and Hentze, 2003).

Finally, the 43S complex binds to the 5`end of the mRNA through interactions of eIF3 with the central part of eIF4G (Macdonald, 2001) and starts to scan the mRNA to the 3`end direction "searching" for an AUG start codon in an optimal context (Kozak, 1989). This scanning process requires ATP hydrolysis. Once the AUG codon is recognized, the GTP associated with eIF2 is hydrolyzed to GDP in a reaction that requires factor eIF5. Next, many of the initiation factors are believed to dissociate from the 40S subunit, leaving only the Met-tRNA$_i$ in the P site already base-paired to the AUG codon of the mRNA, completing the initiation phase of protein synthesis. Next, the factor eIF5B facilitates the joining of the 60S ribosomal subunit. Once the 60S subunit binds the 40S subunit, a complete 80S ribosome is formed, and translation elongation begins (Nielsen et al., 2004). The factor eIF5B in turn hydrolyses its GTP and is released from the 80S ribosome. The 80S ribosome carrying the tRNAs has the important task to scan the mRNA adding the corresponding anticodons until it reaches a stop codon and protein synthesis is terminated (Figure 1.2) (Preiss and Hentze, 2003).

## 1.4. Perfect start codon

Specific sequences modulate the efficiency of a ribosome to recognize an AUG start codon during the scanning process. In vertebrate mRNAs, the initiation site usually compromises all or part of the sequence GCCRCC**AUG**G. The most highly conserved base in this sequence is the purine, usually A, at

position -3. The counting starts at AUG, where the adenosine in the AUG (bold) is designed +1 (Kozak, 1986; Kozak, 1989; Kozak, 1991c). The G in position +4 is highly conserved especially in the absence of A in the position -3 (Kozak, 1997). As long as positions -3 and +4 follow the so called Kozak rule, the rest of the motif (GCC) seems to be less important. However, in the absence of the important bases in positions -3 and +4, the upstream GCC motif strongly contributes to the efficiency of a ribosome to recognize the start codon (Kozak, 1987b).

## 1.5. Cap-Independent Translation

As mentioned previously, the cap structure located at the 5`end of the mRNA has an important role recruiting the 40S ribosomal subunit through interactions with initiation factors. Consequently the mechanism of translation is dependent on the cap structure, and therefore, is called cap-dependent mechanism. Cap-dependent translation is not the only way to translate mRNAs. Translation initiation by internal ribosome entry is an alternative mechanism whereby the 40S ribosomal subunit binds to the mRNA at or near the authentic AUG codon, independent of the cap structure, and therefore enables these mRNAs to be translated by a so called cap-independent translation.

The best understood example of cap-independent translation occurs by certain picornaviruses. The picornavirus family includes several pathogens of both humans (e.g., poliovirus, coxsackievirus, and hepatitis A viruses) and animals (e.g., foot-and-mouth disease virus and encephalomyocarditis virus) (Hellen and Sarnow, 2001; Zamora et al., 2002). Recently, it has been demonstrated that also cellular mRNAs can be translated by a cap-independent mechanism. This mechanism could be advantageous in situations where cap-dependent translation is inhibited, e.g. during apoptosis or hypoxia. During apoptosis activated caspases cleave several initiation factors, including eIF4G, inhibiting in this way the cellular cap-dependent translation (Marissen and Lloyd, 1998). In this situation it could be imagined

that some mRNAs encoding important proteins for these processes would be translated by a cap-independent mechanism.

The initiation factors involved in translation of mRNAs mediated by internal ribosome entry are still not well characterized. The 5`end of picornavirus family mRNAs have a 5`-pUp terminus, instead of the common 5`-cap structure (Nomoto et al., 1977). This peculiar feature suggests that translation of this mRNA does not require the eIF4E, because the function of this initiation factor is to bind to the cap-structure and together with eIF4F complex recruit the ribosome to the cap structure. Indeed inhibition of eIF4E did not alter IRES-dependent translation and thus confirmed this hypothesis (Canaani et al., 1976; Pause et al., 1994). The fact that picornavirus rapidly inhibits translation of capped cellular mRNAs in infected cells support this finding (Macejak and Sarnow, 1991; Sarnow, 1989; Yang and Sarnow, 1997). When mammalian cells are infected by picornavirus, the factor eIF4G is cleaved by action of virus-encoded proteinases $2A^{pro}$ (entero- and rhinovirus) or $L^{pro}$ (aphthovirus) (Haghighat et al., 1996; Lamphear and Rhoads, 1996; Ventoso et al., 1998). Cleavage of eIF4G preclude infected cells from using the cap-dependent initiation mechanisms, instead of that, viral translation proceeds via cap-independent mechanism that require a cis-acting internal ribosome entry site (IRES) on the mRNA (Gan et al., 1998). In this way, eIF4G mediated the shut down of cap-dependent mechanisms and in turn activated cap-independent mechanisms (Gan et al., 1998).

In order to confirm that translation of RNAs from these viruses occur in a cap-independent manner, the 5`UTR of EMCV and poliovirus were placed into the intercistronic spacer region of a bicistronic mRNA. These sequences were able to support translation of the second cistron independent of translation of the fist cistron. This discovery demonstrated that these virus 5`UTRs contain an IRES and can be translated independently of the cap-structure (Jang et al., 1988; Jang et al., 1990; Jang and Wimmer, 1990; Pelletier and Sonenberg, 1988). *BiP*, the gene that codes for the immunoglobulin heavy chain binding protein, was the first cellular mRNA described to contain an IRES, and was found in cells infected by poliovirus,

where translation of the host mRNA was inhibited (Macejak and Sarnow, 1991; Sarnow, 1989; Yang and Sarnow, 1997). After this discovery, several other cellular mRNAs have been reported to contain an IRES by application of the bicistronic assay, e.g., antennapedia (Ye et al., 1997), fibroblast growth factor 2 (Vagner et al., 1995), platelet-derived growth factor B (PDGF2/c-sis) (Bernstein et al., 1997), vascular endothelial growth factor (Miller et al., 1998; Stein et al., 1998), insulin like growth factor II (Teerink et al., 1995), the transcription repressor *Mnt* (Stoneley et al., 2001), the X-chromosome linked inhibitor of apoptosis *XIAP* and *c-myc* (Nanbru et al., 1997). Interestingly, many translation products of cellular mRNAs described to contain an IRES play a role in growth control.

Although IRES mediated translation is independent from some initiation factors, including eIF4E, footprinting and mutagenesis analysis have recently demonstrated that the eukaryotic factors 4G and 4A mediate conformational changes downstream of the initiation codon of the EMCV IRES. This process is ATP-independent and is used to prepare a site on the IRES to which the ribosome can bind efficiently (Kolupaeva et al., 2003). In fact, it has been suggested that internal initiation in picornavirus translation requires all canonical eukaryotic initiation factors that are also used for a cap-dependent translation mechanism, except for eIF4E (Lomakin et al., 2000; Pestova et al., 1996; Pilipenko et al., 2000). In addition, five cellular *trans*-acting factors (ITAFs) specific for picornavirus IRES mediated translation have also been identified, four of them are RNA-binding proteins:

(a) The polypyrimidine tract-binding protein (PTB)(Kolupaeva et al., 1996; Niepmann et al., 1997).

(b) The poly (rC)-binding protein 2 (PCBP2)(Blyn et al., 1996; Gamarnik and Andino, 2000; Walter et al., 1999).

(c) The autoantigen La (Costa-Mattioli et al., 2004; Holcik and Korneluk, 2000; Kim et al., 2001; Ray and Das, 2002).

(d) The RNA binding protein Unr (Boussadia et al., 2003; Brown and Jackson, 2004; Hunt et al., 1999; Mitchell et al., 2003).

(e) The cell cycle dependent protein $ITAF_{45}$ (Pilipenko et al., 2000).

Functional *in vitro* assays showed that a combination of two or three of these ITAFs are required for a cap-independent IRES mediated translation initiation (Pilipenko et al., 2000). In *XIAP* mRNA, for example, analysis by UV cross-linking experiments demonstrated that the autoantigen La is an essential part of the XIAP IRES ribonucleoprotein (RNP) complex. However PTB and PCBP do not bind to this complex (Holcik and Korneluk, 2000). In addition, other ITAFs were identified that bind to the XIAP IRES, i.e. two heterogeneous nuclear ribonucleoproteins (hnRNPC1 and C2) (Holcik et al., 2003). These ribonucleoproteins are known to be involved in different steps of RNA biogenesis, including splicing (Choi et al., 1986), RNA turnover (Hamilton et al., 1993) and polyadenylation (Wilusz and Shenk, 1990). It has been reported that the hnRNPC1 and hnRNPC2 do not only mediate IRES-dependent XIAP translation, but has also been shown to bind to the IRESes of PDGF/c-sis (Sella et al., 1999) and p27 mRNAs (Kullmann et al., 2002; Millard et al., 2000). Further evidence for additional cellular proteins being important for IRES utilization is provided by the observation that IRES containing cellular mRNAs do not function properly in a cell-free translation system or in RNA transfection assays, suggesting that these identified IRESes need a nuclear event (Iizuka et al., 1995; Stoneley et al., 2000).

Interestingly the 5` UTR of the identified mRNAs that are translated by a cap-independent mechanism, are long, contain uAUG codons, and are G-C rich forming stable secondary structures that may block ribosome scanning during cap-dependent translation.

## 1.6. Assays used to determine IRES activity

Several types of assays are widely used to analyze whether a specific sequence harbors an IRES. The bicistronic assay is considered to be the gold standard (Figure 1.3). In this assay, the sequence under scrutiny is placed in an intercistronic region between two reporter genes, and transiently transfected into cells. The DNA is transcribed into RNA using a promoter located upstream of the first cistron. If the sequence does not contain an IRES,

the ribosome will leave the mRNA after translation of the first reporter gene (Figure 1.4 pathway 1). However, if the inserted sequence contains an IRES, translation of the second cistron will be mediated by the IRES, and activities of both reporter genes will be measured. The critical question in this kind of assay is whether the inserted sequence stimulates the expression of the second cistron to a level comparable to that of the first cistron. Cases where the downstream cistron is translated at a level less than 10% of the expression of the first cistron should be considered with some suspicion (Jackson et al., 1995).

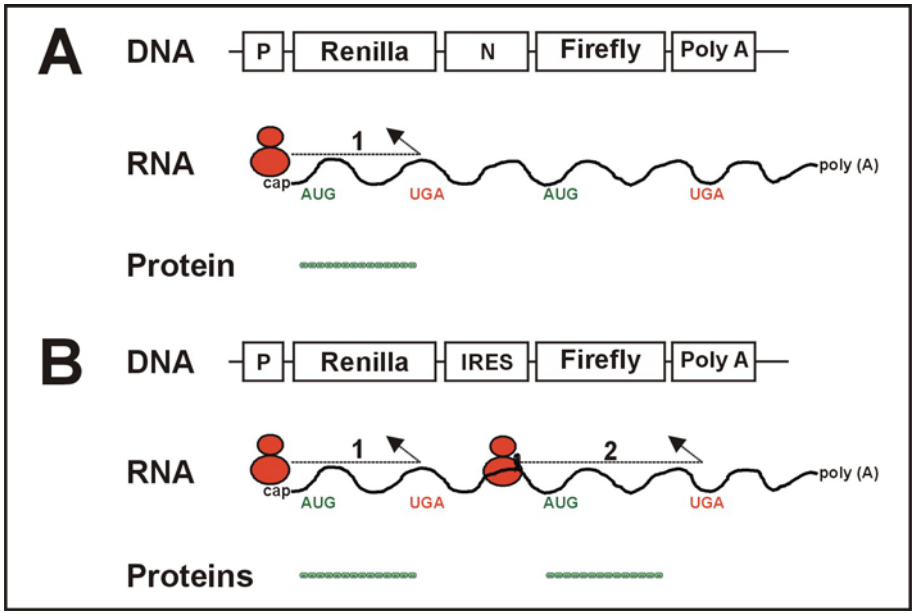



**Figure 1.3. Cap dependent and IRES-dependent translation.**
The vector contains two open reading frames: Renilla luciferase (RL) and Firefly luciferase (FL), respectivly. The transcription is driven by the promoter located upstream the RL cistron. The transcribed RNA contains a cap-structure and a poly (A) tail. In order to start protein synthesis, the 40S ribosomal subunit associates with transcription factors, recognizes the cap-structure and scans the mRNA until the AUG codon of the RL cistron is encountered. After translation of RL, the ribosome dissociates from the mRNA. The FL cistron is not translated. (B) The DNA contains an IRES sequence upstream the FL cistron. Transcription is still driven by the promoter encountered upstream the RL cistron. In this situation, two proteins are produced from only one mRNA. This is possible because the ribosome still can bind to the cap-structure and translate the RL cistron (pathway 1). In addition, the ribosome can also bind to the IRES sequence, and start translation of the FL cistron (pathway 2). The complete 80S ribosome is depicted in red. AUG and UGA corresponds to start and stop codon, respectively.

It is also common to place an inverted sequence upstream of the first cistron. This sequence which forms a stable hairpin when transcribed has the power to block ribosome scanning, and therefore abolishes expression of the

first cistron (Bernstein et al., 1997; Blaschke et al., 2003). In contrast, expression of the second cistron should not be severely altered if the intercistronic sequence contains an IRES. The addition of a hairpin without careful analysis of the mRNA can still fail to prove that an inserted sequence contains IRES activity, because the presence of cryptic promoter or splice sites in the sequence under investigation may interfere with this assay. For that reason, the sequence under scrutiny should pass other control assays. For example, one should verify whether the putative IRES can function in an *in vitro* assay. In this assay, the DNA is transcribed using a T3 or T7 promoter *in vitro* followed by translation using a rabbit reticulocyte lysate. If the sequence analyzed contains an IRES, the second cistron should be translated. Unfortunately the interpretation of the results may be hampered by two different observations. 1) It has been described that the reticulocyte lysate system may initiate translation at a number of incorrect sites on some mRNAs (Borman and Jackson, 1993; Dorner et al., 1984). 2) It is also possible that ITAFs that are required for a perfect IRES function are not present in the rabbit reticulocyte lysate. This possibility was described for poliovirus and rhinovirus IRESes which were not active in an *vitro assay* unless the lysate was supplemented by HeLa cell extracts (Borman et al., 1993; Brown and Ehrenfeld, 1979; Dorner et al., 1984).

Another assay should be performed to verify whether the putative IRES encompass a cryptic promoter. For this purpose, one should simply remove the promoter of the construct and look for reporter gene activity. If the sequence does not contain a cryptic promoter, no transcription should occur and no translation of the reporter gene should be measured. If the sequence under investigation encloses a promoter sequence, transcription and subsequently translation will occur. In this case the presence of the promoter may be detected by Northern Blot analysis. However, the promoter has to be strong enough to be detected by this kind of analysis. It has been reported that the level of translation of the second cistron should be at least 5% of that of the first cistron, in order to be able to observe the transcript by Northern blot analysis (Kozak, 2003).

Transfection of the bicistronic mRNA directly into the cells has also been performed to confirm the presence of an IRES in the sequence investigated. In this assay, the RNA is transcribed *in vitro* and directly used for transient transfection into cells. Expression of the second cistron should occur.

## 1.7. Leaky scanning

Translation initiation usually starts at an AUG codon that is near the mRNA 5` end, and is embedded in an optimal "environment" represented by the nucleotide sequence GCCRCC**AUG**G (section 1.4). In this situation all or almost all ribosomes stop scanning and initiate protein synthesis on that AUG. However, when the AUG resides in a very weak context, lacking both, R in position -3 and G in position +4, some ribosomes may initiate at that point but most continue scanning and initiate farther downstream. This process is called leaky scanning and allows producing two proteins from only one mRNA (Kozak, 2002b). A list of examples can be found in Table 3 of Kozak, M (Kozak, 2002b). There are also rare cases of leaky scanning where the first AUG resides in a good context ($R^{-3}$ and $G^{+4}$) that is bypassed by the ribosome which starts at a downstream AUG. This may occur when the first AUG codon is too close to the 5`end to be recognized efficiently (Kaneda et al., 2000; Kozak, 1991b; Slusher et al., 1991; Spiropoulou and Nichol, 1993; Werten et al., 1999) or when the positive effect of the G in position +4 is counteracted by an U in position +5 (Kozak, 1997; Sloan et al., 1999; Stallmeyer et al., 1999). There are mammalian mRNAs however that completely lack a start codon in an optimal context (Arai et al., 1991; Hickey and Roth, 1993; Leslie et al., 1992; McNeil et al., 1992; Plowman et al., 1990; Wu et al., 1993). A good example of leaky scanning is the *c-myc* gene. The *c-myc* gene produces three proteins from only one mRNA. The first start codon has the sequence AGG**CUG**G, producing a 68 kDa long protein. The second start codon has the sequence ACG**AUG**C, leading to a protein of 65 kDa, which is the predominant form of the C-MYC *protein*. The last start codon has the sequence GAG**AUG**A,

leading to a protein of 50 kDa. Only a small amount of long protein is translated (68 kDa), because its start codon is not an AUG. The second start codon contains an AUG and even an A in position -3, however still some ribosomes bypass that site and initiate at the next AUG. This can happen because the first AUG is in a good but still not perfect context. This was confirmed by mutagenesis analysis, where the first AUG was changed into a perfect start codon. In this case the small protein was not detectable (Spotts et al., 1997). When discussing leaky scanning, one should also mention the process of ribosome reinitiation in which the ribosome may resume scanning of the mRNA after translation of a small peptide. This matter will be discussed in more details in the section about upstream open reading frames (section 1.9).

## 1.8. Ribosome shunting

Ribosome shunting is the mechanism in which ribosomal subunits bind to the mRNA in a 5`cap-dependent manner and scan downstream until a stable RNA structure is encountered that may arrest the scanning ribosome or cause its dissociation from the mRNA. This arrest is followed by intramolecular shunting of the ribosomal subunit to a downstream site, bypassing in this way some of the RNA structure, and the ribosome resumes scanning until the next appropriate start codon is reached (Hellen and Sarnow, 2001).

## 1.9. Upstream open reading frame

Usually the 5`UTR sequence of the mRNA is short and the mRNA itself contains only one open reading frame. In fact, less than 10% of cellular mRNAs have a long 5` UTR and contain one or more upstream AUG (uAUG) codons. The uAUG codons often create small open reading frames (uORFs). (Kozak, 1987a). Although uORFs are rare they represent a common feature of genes involved in the control of cellular growth and differentiation (Kozak, 1987a; Kozak, 1991a; Morris, 1995), including two-third of the oncogenes.

uORFs usually exhibit three potential configurations following uAUG codons (Figure 1.4) (Geballe and Sachs, 2000):

1) **Nonoverlapping** – a nonoverlapping uORF is the one which has the termination codon located in the 5`UTR.
2) **Overlapping** – an overlapping uORF has its termination codon located somewhere in the main ORF.
3) **In frame** – in frame is the uORF that is in frame with the main ORF and ends at the same termination codon.

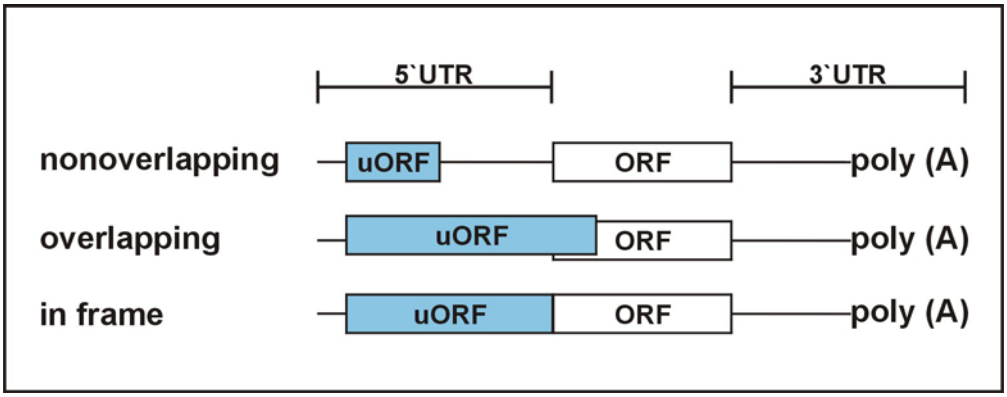



**Figure 1.4. Three possible configurations of ORFs following upstream AUG codons.**

Features that regulate protein synthesis of any uORF, seems to be identical to those described for major ORFs, e.g. the context of its start codon ($R^{-3}$ and $G^{+4}$)(section 1.4); its secondary structure and the proximity of the AUG codon to the cap structure (Cao and Geballe, 1995; Kozak, 1999; Ruan et al., 1994; Wang and Sachs, 1997; Werner et al., 1987).

Unfortunately, the signals that are responsible for the mechanisms involved in reinitiation or continued scanning after translation of the uORF are not completely understood. Some possibilities for the ribosome after translation of the uORF are described below:

**1) The ribosome dissociates from the mRNA** – one possibility is that the ribosome may encounter structures that facilitate the dissociation from the mRNA, or the ribosome may leave the mRNA just because the ribosome has finished already its function in translating an uORF. In this case, translation of

the downstream AUG would not occur, and this uORF would have the function of blocking protein translation of the downstream ORF.

**2) The ribosome remains associated with the mRNA** – another possibility is that the ribosome remains associated with the mRNA, continues scanning, and reinitiates at either a proximal or distal AUG codon (Cao and Geballe, 1995; Mize et al., 1998). The problem in this situation is that the ribosome may need to reacquire some initiation factors, especially eIF2-GTP which carries the Met-tRNA$_i$ in order to reinitiate at a downstream AUG codon (Hinnebusch, 1996). In agreement with that, a longer "spacing region" between the stop codon of the uORF and the following start codon would result in a higher probability of the ribosome to reacquire the necessary initiation factors (Kozak, 1998). This mechanism has been confirmed for translation of the *GCN4* mRNA by demonstrating that an increase of the intercistronic space to up ~80 nucleotides reduces or eliminates the inhibitory effect of the uORF in some cases (Child et al., 1999; Kozak, 1987c).

**3) The ribosome stalls** – the ribosome may also stall after translation of the uORF, or even during elongation, thereby blocking protein translation by prohibiting the following ribosome from continued scanning. This has been observed in *S. cerevisiae CPA1* and *Neurospora crassa arg-2* uORFs (Wang et al., 1998; Wang et al., 1999). Interestingly, ribosome stalling after translation of the *arg-2* and *CPA1* uORF even after removal of the termination codons of the respective uORFS was observed (Wang et al., 1998; Wang et al., 1999).

**4) mRNA stability** – mRNAs harboring imperfect messages containing premature translational stop codons are recognized and degraded by a process called nonsense-mediated decay (Belgrader et al., 1994; Cheng and Maquat, 1993; Whitfield et al., 1994). Transcripts containing uORFs as a result of mutations are sensitive to NMD. However, most described wild type uORF transcripts are not degraded by this pathway (Ruiz-Echevarria et al., 1998; Vilela et al., 1998), because they have stabilizer elements (STEs) that are able

to inactivate the NMD pathway (Ruiz-Echevarria and Peltz, 2000). However, the yeast *CPA1* uORF mRNA is degraded via the NMD (Ruiz-Echevarria and Peltz, 2000). The NMD machinery is conserved from yeast to humans (Couttet and Grange, 2004). However, the premature termination codon transcripts found in Drosophila are degraded by a mechanism that differs from the degradation mechanism found in yeast and mammals (Cao and Parker, 2003; Lejeune et al., 2003; Mitchell and Tollervey, 2003).

In summary, the examples mentioned above clearly demonstrate that upstream open reading frames regulate biosynthesis by interference and/or regulation of translation efficiency. Some examples concerning the relation between uORFs and diseases are find in the discussion section.

## 1.10. Objective of this work

Overexpression of the *myeov* gene in a subset of t(11;14) positive multiple myeloma cell lines and esophageal squamous cell carcinomas, and its localization in a critical chromosomal region suggest that *myeov* may play a role in carcinogenesis. At the same time we observed *myeov* amplification and overexpression on an mRNA level in different cells without being able to detect any MYEOV protein. Special features of the *myeov* 5`UTR, like its length of ~450 nucleotides, the presence of four uAUGs encoding four uORFs and its high GC content enabling it to form a highly stable secondary structure, might be an explanation for this phenomenon. We therefore conducted further studies to characterize the *myeov* gene with the following objectives: 1) a search for MYEOV interaction partners in order to be able to elucidate the biological function of the MYEOV protein, 2) characterization of its 5`UTR sequence in order to delineate the mechanisms involved in the regulation of MYEOV biosynthesis.

# 2. Materials and Methods

## 2.1. Materials

### 2.1.1. Equipment

| | |
|---|---|
| Bio-freezer (-80°C) | Forma Scientific |
| Biofuge pico | Heraeus |
| Certoclav (autoclave) | LGA |
| Contact thermomether MR 3002 | Heidolph |
| Digital graphic printer | Sony |
| DNA sequencer long read 4200 and 4300 | Li-cor |
| E. coli pulser DNA electroporator | Bio-Rad |
| Easy cast electrophoresis | Angewandte Gentechnologie |
| Electophoresis camera | Bethesda Research |
| Epichemi II darkroom | UVP |
| Galaxy mini centrifuge (for PCR tubes) | Merck |
| Icemaker B-3905 | Scotsman |
| Incubator (cell culture) | Heraeus |
| Incubator shaker model G25 | New Brunswick Scientific |
| Lucy 2 (Luciferase assay) | Rosys Anthos |
| Mettler delta 320 (pH meter) | Mettler Toledo |
| Microflow biological safety cabinet | Intermed Nunc |
| Microscope 090-131-001 | Leica |
| Microwave oven 9023 | Privileg |
| Mini centrifuge | National Labnet Co. |
| Optimax X-ray film processor 1170-1-0000 | Protec Medizintechnik |
| Orbital shaker | Forma Scientific |
| Peltier thermal cycler PTC-200 | MJ Research |
| Power pac 3000 (power supply) | Bio-Rad |
| Precision weigher FA 3100-2iCE | Faust |
| Reax 2000 (vortex) | Heidolph |
| Refrigared centrifuge RC-5B | Sorvall |
| Refrigared centrifuge RC-5B plus | Sorvall |
| Refrigerated centrifuge 5417 | Eppendorf |
| Sealboy 235 | Audion Elektro |
| Speed vac | Savant |
| Superspeed centrifuge | Du Pont Instruments |
| Thermomixer 5436 | Eppendorf |
| Ultrospec 2000 (spectrophotometer) | Pharmacia Biothech |
| UV stratalinker 2400 | Stratagene |
| UV table foto prep I | Fotodyne |
| Varifuge 3.9 R | Heraeus |
| Vortexer reax 2000 | Heidolph |
| Water bath type 1012 | Faust |
| Wide mini sub cell | Bio-Rad |
| X-cell sure lock | Invitrogen |

## 2.1.2. Chemicals

| | |
|---|---|
| 2- Mercapto-ethanol | Roth |
| 3-Aminophthalhydrazide; Luminol | Sigma |
| Ammonium acetate | Applichen |
| Ammonium chloride | Merck |
| Ammonium persulfate | Sigma |
| Ammonium sulfate | Sigma |
| Ampicillin | Sigma |
| Aqua ad injectabila | Braun |
| Bacto® trypton | BD |
| Bacto® yeast extract | Difco |
| Bromophenol blue | Schmid |
| Calf intestine phosphatase | Roche |
| Coomasie blue R250 | Merck |
| Diethylpyrocarbonat (DEPC) | Roth |
| DMEM high glucose | PAA |
| DMEM/Ham´s F-12 (1x) | PAA |
| DMSO | J.T. Baker |
| dNTPs | MBI |
| DTT | Sigma |
| ECL® | Amersham |
| Ethanol absolut | Riederl-de-Haen |
| Ethidium bromid | Merck |
| Ethylenediamine-tetraaceticacid | Roth |
| Ficoll 400 | Pharmacia |
| Fetal calf serum | PAA |
| Formaldehyde solution | J.T. Baker |
| Formamide solution | J.T. Baker |
| GenRuler® 100 bp and 1 Kb DNA ladder | MBI |
| Glycerol | Roth |
| Glycogen | Roche |
| HEPES | Roth |
| Imidazol | Serva |
| IPTG (isopropyl-β-D-1-thiogalactopyranosid) | Sigma |
| Long ranger® gel solution | BMA |
| Methanol | Merck |
| Nonfat dry milk | Nestlé |
| NZY broth | Gibco BRL |
| Orange G | Sigma |
| PBS | PAA |
| Phenol/chloroform/isoamylalcohol 25:24:1 | Roth |
| Ponceau S solution | Sigma |
| Potassium acetate | Gerbu |
| Potassium chloride | Riedel-de-Haen |
| Propanol | Merck |
| Proteinase K | Sigma |
| Rothiphorese® gel 40 (29:1) | Roth |

| | |
|---|---|
| SDS | Roth |
| SeeBlue® Plus2 prestained standard | Invitrogen |
| Select agar | Invitrogen |
| Spermidin | Sigma |
| TEMED | Pharmacia |
| Tetracycliin | Sigma |
| Tris | Sigma |
| Triton X-100 | Fluka |
| Trypsin/EDTA (1x) | PAA |
| Tween 20 | Roth |
| Urea | Pharmacia |
| X-Gal | Roth |
| Yeast extract | Difco |

## 2.1.3. Buffers

| | | | |
|---|---|---|---|
| **BBS** | 50 | mM | BES/HCl, pH 6.95 |
| | 280 | mM | NaCl |
| | 1.5 | mM | $Na_2HPO_4$ |
| **Bloting (10 x)** | 47.9 | mM | Tris/HCl, pH 6.8 |
| | 38.6 | mM | Glycine |
| | 0.037 | % | SDS |
| **CIP (10 x)** | 100 | mM | Tris/HCl, pH 8.0 |
| | 10 | mM | $MgCl_2$ |
| | 10 | mM | $ZnCl_2$ |
| **Denaturation buffer** | 0.5 | M | NaOH |
| | 1.5 | M | NaCl |
| **Denhardt´s (100 x)** | 2 | % | Ficoll |
| | 2 | % | BSA Fraktion V |
| | 2 | % | Polyvinylprolidon type 360 |
| **Electroporation (5 x)** | 125 | mM | Tris/HCl, pH 6.8 |
| | 1.25 | mM | Glycine |
| | 0. 5 | % | SDS |
| | 20 | mM | EDTA |
| **Hybridization mix** | 10 | X | Denhardt´s |
| | 10 | % | Dextran sulfate |
| | 1 | % | SDS |
| | 3 | X | SSC |
| | 50 | mg/l | Salm DNA |

| | | | |
|---|---|---|---|
| **SSC (20 x)** | 3 | M | NaCl |
| | 0.3 | M | Sodium citrate, pH 7.0 |
| **SSC wash mix (3 x)** | 3 | X | SSC |
| | 0.1 | % | SDS |
| **SSC wash mix (0.3 x)** | 0.3 | X | SSC |
| | 0.1 | % | SDS |
| **TBE (10 x)** | 0.9 | M | Tris |
| | 0.9 | M | Boric acid |
| | 20 | mM | $Na_2EDTA$, pH 8,3 |
| **TBS** | 10 | mM | Tris/HCl, pH 7,4 |
| | 150 | mM | NaCl |
| **TBS-T** | TBS + | 0.05% | Tween 20 |
| **TE** | 10 | mM | Tris/HCl, pH 7.5 |
| | 1 | mM | EDTA |
| **10 x Ligase** | 500 | mM | Tris/HCl, pH 7.8 |
| | 100 | mM | $MgCl_2$ |
| | 100 | mM | DTT |
| | 10 | mM | ATP |
| | 500 | µg/ml | BSA |
| **Neutralization** | 0.5 | M | Tris/HCl, pH 7.5 |
| | 3 | M | NaCl |
| **Ponceau S** | 0.2 | % | Ponceau S |
| | 3 | % | Tri-Cl-acetic acid in water |

## 2.1.4. Enzymes

| | |
|---|---|
| Calf intestine phosphatase | Roche |
| *Pfu* DNA polymerase | Promega |
| RNase A | Roche |
| T4 DNA ligase | Promega |
| Taq DNA polymerase | Gibco |

*All restriction endonucleases inclusive the respective buffers were purchased from New England Biolabs, Roche and MBI Fermentas.*

## 2.1.5. Special materials

| | |
|---|---|
| 96-well plate (for luciferases measurements) | Nunc |
| BioMax MS film | Kodak |
| BioMax MS intensifying | Kodak |
| BioMax XAR film | Kodak |
| Cell culture flasks and plates | Greiner |
| Disposable filters unit: 0.2 and 0.45 µm | Schleicher & Schuell BioScience |
| Electroporation cuvette 1.0 mm | Equibio and Bioenzym |
| Falcon tube 2059 | Becton Dickinsons |
| Folded filters 240 mm | Schleicher & Schuell BioScience |
| Hematocytometer 0.0025 $mm^2$ | Brand |
| Hybond™-N | Amersham Biosciences |
| Hypercassete | Amerscham Biosciences |
| Precision quartz cuvette | Hellma |
| Precision wipe tissue | Kimberly-Clark |
| Protein gel cassette 1.0 mm | Invitrogen |
| Protran® nitrocellulose membrane | Schleicher & Schuell BioScience |

## 2.1.6. Special reagents and kits

| | |
|---|---|
| Cycle Reader™ Auto DNA Sequencing Kit | MBI Fermentas |
| DNA Gel Extraction Kit | Montage |
| Dual Luciferase Reporter Assay System Kit | Promega |
| ECL Kit | Amerscham Biosciences |
| Fugene | Roche |
| Gene Juice® Transfection Reagent | Novagen |
| Genelute™ Direct Mrna Miniprep Kit | Sigma |
| Genelute™ HP Plasmid Maxiprep Kit | Sigma |
| Hexalabel™ DNA Labeling Kit | MBI Fermentas |
| High Pure RNA Isolation Kit | Roche |
| Mmessage Mmachine™ | Ambion |
| Mrna Isolation Kit | Roche |
| PCR Polishing | Stratagene |
| Pgem® - T Easy Vector System I | Promega |
| QIAEX® II Gel Extraction Kit | Qiagen |
| Qiafilter™Plasmid Maxi Kit | Qiagen |
| Quikchange® Multi Site-Direct Mutagenesis | Stratagene |
| Rneasy Minelute Cleanup Kit | Qiagen |
| TNT® Coupled Reticulocyte Lysate Systems | Promega |
| Transmesseger™Transfection Reagent | Qiagen |

## 2.1.7. Bacterial strains

| Strain | Genotype |
|---|---|
| **DH10B** | F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*Δ139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ- rpsL (Str$^R$) *nup*G |
| **DH5α** | F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17($r_k^-$, $m_k^+$) *pho*A *sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1 |
| **TOP10** | F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K rpsL (Str$^R$) *end*A1 *nup*G |
| **XL10 – Gold** | TetrΔ (mcrA) 183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte (F`proAB laclqZΔM15Tn10 (Tetr) Amy Camr)a |
| **XL-2 Blue** | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F`proAB laclqZΔM15Tn10 (Tetr) Amy Camr)a |

## 2.1.8. Cultivation

*E. coli* was cultivated overnight in Luria Bertani medium (LB-medium) at 37°C under vigorous shaking. When required, antibiotic was added to the medium in order to make selection (Table 2.1).

**Table 2.1. Stock and working solution of antibiotic**

| Antibiotic | Stock solution concentration | Working concentration |
|---|---|---|
| Ampicillin | 50 mg/ml in water | 100 µg/ml |
| Chloramphenicol | 34 mg/ml in ethanol | 34 µg/ml |
| Kanamycin | 10 mg/ml in water | 50 µg/ml |
| Tetracyclin | 15 mg/ml in ethanol | 15 µg/ml |

**LB medium** was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 800 ml distilled water. The pH was adjusted to 7.5 with NaOH and distilled water was added to a final volume of one liter. The LB medium was sterilized by autoclaving at 120°C for 15 minutes, and stored at room

temperature. In order to make selection media, antibiotic was added to the LB medium according to the concentration described in Table 2.1, and the antibiotic containing LB was stored at 4°C.

**LB-agar plate** was prepared by adding 15 g/l of select agar to LB medium and sterilized by autoclaving as described above.

**NZY broth** was prepared by dissolving 10 g NZ amine (casein hydrolysate), 5 g yeast extract and 5 g NaCl in 800 ml distilled water, pH was adjusted to 7.5 with NaOH, and water was added to a final volume of 1 liter. Prior to use 12.5 ml/l 1 M $MgCl_2$, 12.5 ml/l $MgSO_4$ and 10 ml/l of filter-sterilized 2 M glucose were added.

**Freezing** - Bacteria grown on LB-media were frozen by adding one tenth volume of 10 x Hogness Freezing Medium (Werner et al., 1997) to 0.9 ml aliquot of bacteria and stored at -80°C.

| **10x Hogness Freezing Medium** | | |
|---|---|---|
| $K_2HPO_4$ | 360 | mM |
| $KH_2PO_4$ | 132 | mM |
| $MgSO_4$ x 7 $H_20$ | 4 | mM |
| $Na_3$-citrate x 2 $H_20$ | 17 | mM |
| $(NH_4)_2SO_4$ | 68 | mM |
| Glycerol (v/v) (autoclaved) | 44 | % |

## 2.1.9. Oligonucleotides primers

## 2.1.9.1. To study an Internal Ribosome Entry Site (IRES)

| | |
|---|---|
| **myeovEcoRIUTRfor** | 5`- CGGAATTCGAACCCACATCCCTACAAAGCAG -3` |
| **myeov14NcoIUTRrev** | 5`- CATGCCATGGGCCGAGGGAAGGAGCCAG -3` |
| **myeov14NcoIUTRrevA** | 5`- CATGCCATGG**T**CCGAGGGAAGGAGCCAG -3` |
| **myeovUTREcoRVrev** | 5`- GGTTCCGATATCGAGCCGAGGGAAGGAGCC -3` |
| **myeovUTRHindIIIfor** | 5`- CAGCCCAAGCTTCGGACCGCGAACCCACATC -3` |

## 2.1.9.2. To study uORFs

| | |
|---|---|
| **myeovATG1*** | 5`- CAAAGCAGGAA**A**GTAAGCTTGGGAGAGGCC -3` |
| **myeovATG2*** | 5`- CAGAGGGCGGGAGA**A**GCCATCCCCACTG -3` |
| **myeovATG3*** | 5`- GGGCCGGGGCGTGCA**A**GGCCTCAGGG -3` |
| **myeovATG4*** | 5`- GGCCTCAGGGA**A**GGCCTGTTCAGCTGC -3` |
| **HRVirusIRESHindIII*** | 5`- AAGCGCCTACACAAAG**T**TTAGTAGCATCTCCG -3` |
| **EMCVirusIRESHindIII*** | 5`- GCAGTTCCTCTGGAAG**T**TTCTTGAAGACAAGC -3` |
| **myeovUTRHindIIIfor** | 5`- CAGCCCAAGCTTCGGACCGCGAACCCACATC -3` |
| **myeovEcoRVrev** | 5`- GGTTCCGATATCGAGCCGAGGGAAGGAGCC -3` |

***** *Oligonucleotides primers modified in their 5`phosphate group to be used in a mutagenesis assay.*

## 2.1.9.3. Construction of *myeov* cDNA into the vector pMT2SM to study its 5`and 3`UTR

| | |
|---|---|
| **myeovKpnKozaklong-14** | 5`- GGGGTACCGCCACCATGGCCCTCAGAATCTGCG -3` |
| **myeovKpnownlong14** | 5`- GGGGTACCTTCCCTCGGCTCATGGCC -3` |
| **myeovKpnKozakshort2** | 5`- GGGGTACCGCCACCATGGTCACCCGGCAGGCTGGAC -3` |
| **myeovKpnATGown2short** | 5`- GGGGTACCGGGACTCGTTGCTCATGTTC -3` |
| **myeov-Xba-end** | 5`- GCTCTAGATCAACAAGTGAGGATGATGATG -3` |
| **myeov-Xba-L-end** | 5`- GCTCTAGATCAAGGAGAAGCACCTGACACTG -3` |

## 2.1.9.4. Promoter characterization

| | |
|---|---|
| **EcoRImyeov5UTRdel1for** | 5`- CGGAATTCCTTTTTCCCCACCTTGGACAG -3` |
| **EcoRImyeov5UTRdel2for** | 5`- CGGAATTCCACCTGCAGGGCCGGGG -3` |
| **EcoRImyeov5UTRdel3for** | 5`- CGGAATTCCTGCTCGTTGCCTTTGGGC -3` |
| **myeov14NcoIUTRrev** | 5`- CATGCCATGGGCCGAGGGAAGGAGCCAG -3` |

## 2.1.9.5. Transcription factor

| | |
|---|---|
| **myeovBampBindfor** | 5`- CGCGGATCCTGGCCCTCAGAATCTGCGTCAC -3` |
| **myeovXbaEnde** | 5`- GCTCTAGATCAACAAGTGAGGATGATGATG -3` |
| **BamVP16for** | 5`- CGCGGATCCTGTCGACGGCCCCCCCGAC -3` |
| **XbaVP16rev** | 5`- GCTCTAGATCATCCCGGACCCGGGGAATCCC -3` |

### 2.1.9.6. Northern Blotting probes

| | |
|---|---|
| **GAPDHfor** | 5`- TTAGCACCCCTGGCCAAGG -3` |
| **GAPDHrev** | 5`- CTTACTCCTTGGAGGCCATG -3` |
| **Luiferase for** | 5`- GGAGAGCAACTGCATAAGGC -3` |
| **Luciferase rev** | 5`- CATCGACTGAAATCCCTGGT -3` |
| **Renilla luciferase for** | 5`- ATGTTGTGCCACATATTGAGCCAGT -3` |
| **Renilla luciferase rev** | 5`- GATTTCACGAGGCCATGATAATGA -3` |

### 2.1.9.7. Sequencing primers

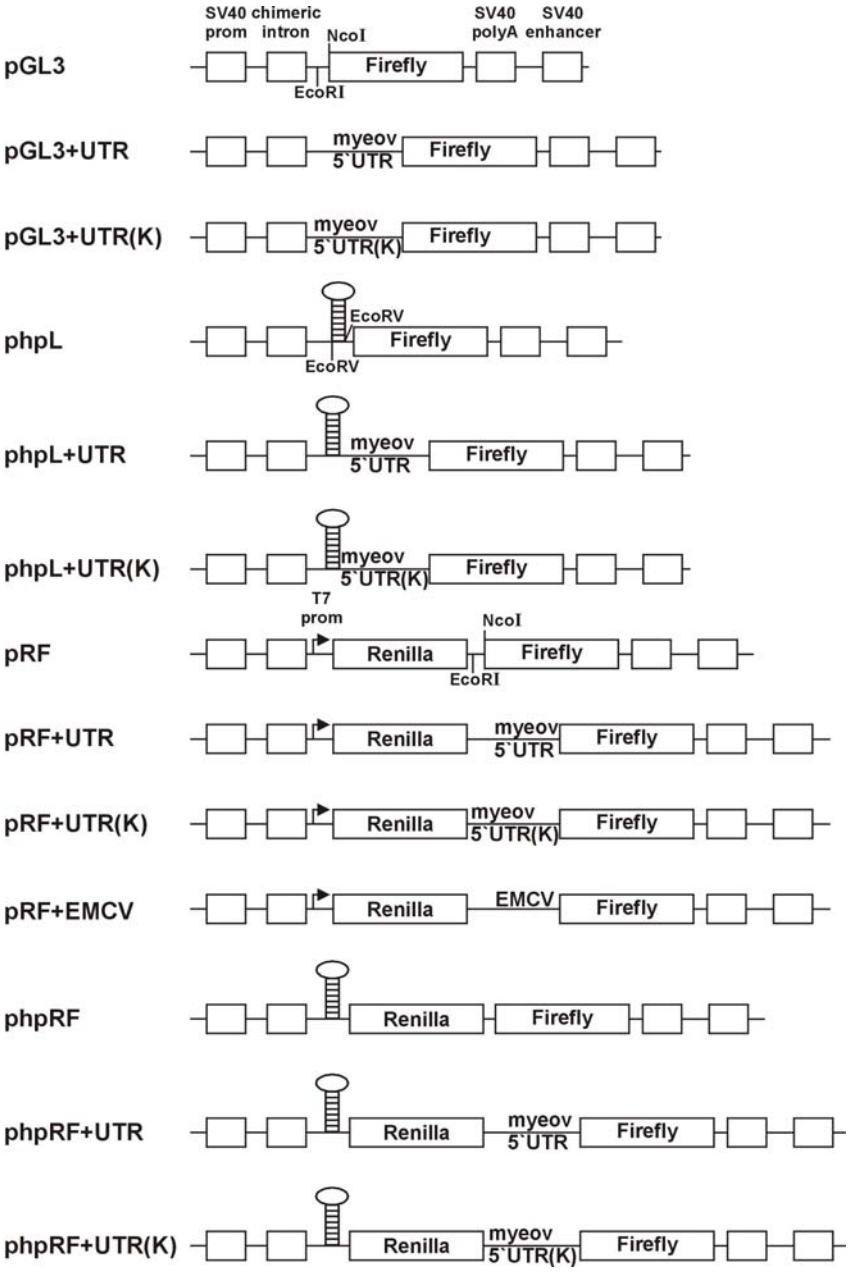
| | |
|---|---|
| **M13for (700)** | 5`-GTAAAACGACGGCCAGT-3` |
| **M13rev (800)** | 5`-CAGGAAACAGCTATGAC-3` |
| **Fireluc (700)** | 5´-CTTCTGCCAACCGAACGGAC-3` |
| **RenillaLucRev (700)** | 5´-ACACCGCGCTACTGGCTC-3` |
| **pMT2Smrev (700)** | 5´-GCGACGATGCAGTTCAATGG-3 |
| **pMT2Smfor (800)** | 5`-GGTGATGCCTTTGAGGGTGG-3` |
| **myeovseq1098rev (700)** | 5`- CCACCGCCCTTGCAGAC-3 |
| **myeovseq882forw (800)** | 5`-GCGGTGAGAGGAGCATTTG-3` |

## 2.1.10. Plasmids

### 2.1.10.1. Establishment of the mono- and bicistronic constructs.

The luciferase plasmids: pGL3, phpL, pRF, phpRF and pRF+EMCV have been described previously and were kindly provided by Dr. A. Willis (Stoneley et al., 2000). *Pfu* DNA polymerase (Promega) was used to amplify the complete *myeov* 5`UTR using a cloned cDNA 11SMNp14m81 encoding the large ORF and the following primers: myeovEcoRIUTRfor and myeov14NcoIUTRrev or myeov14NcoIUTRrevA (used to keep the perfect Kozak start codon of the Firefly luciferase gene). A`s were added by the addition of dATP (Pharmacia) and Taq DNA polymerase and incubation at 70ºC for 30 minutes. The fragment was purified after gel electrophoresis using a gel extraction kit from Qiagen or Montage, and inserted into a T-vector, pGEM-T easy plasmid (Promega) originating pGEM-T+myeov5`UTR and pGEM-T+myeov5`UTR(K). The identities of the inserts were verified by DNA

sequencing using M13-forward and reverse primers (M13for and M13rev). The fragment was digested with EcoRI and NcoI restriction endonucleases and cloned into pGL3, phpL, pRF and phpRF between EcoRI and NcoI sites thus creating pGL3+UTR, pGL3+UTR(K), phpL+UTR, phpL+UTR(K), pRF+UTR, pRF+UTR(K), phpRF+UTR and phpRF+UTR(K). The correct insertion was verified by DNA sequencing using a specifically labelled Firefly luciferase reverse primer (Fireluc) on a Li-Cor 4200 DNA Analyser.

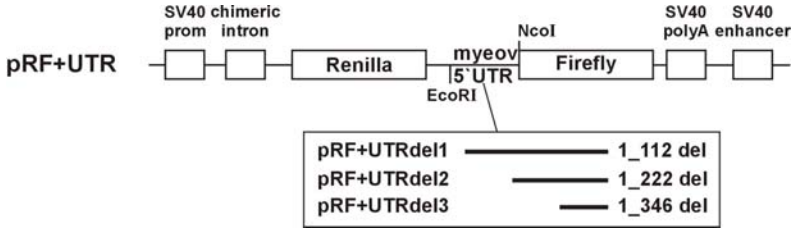


### 2.1.10.2. Promoterless construction

Construction of the mono and bicistronic promoterless constructs were performed by deleting the SV40 promoter sequence between the HindIII and XhoI sites from pGL3 and pGL3+UTR; and the SmaI and EcoRV sites from pRF, pRF+UTR and pRF+EMCV by restriction digestion, agarose gel electrophoresis, purification and religation and thus creating pGL3-P, pGL3-P+UTR, pRF-P, pRF-P+UTR and pRF-P+EMCV, respectively. The pGL3-P+EMCV was construct by digesting the construct pRF+EMCV with SpeI and NcoI restriction endonucleases. The purified fragment (5`UTR of EMCV) was inserted upstream of the Firefly luciferase cistron via SpeI and NcoI sites in the vector pGL3-P. All constructs were checked by sequencing using a specifically labeled Firefly luciferase reverse primer (Fireluc).

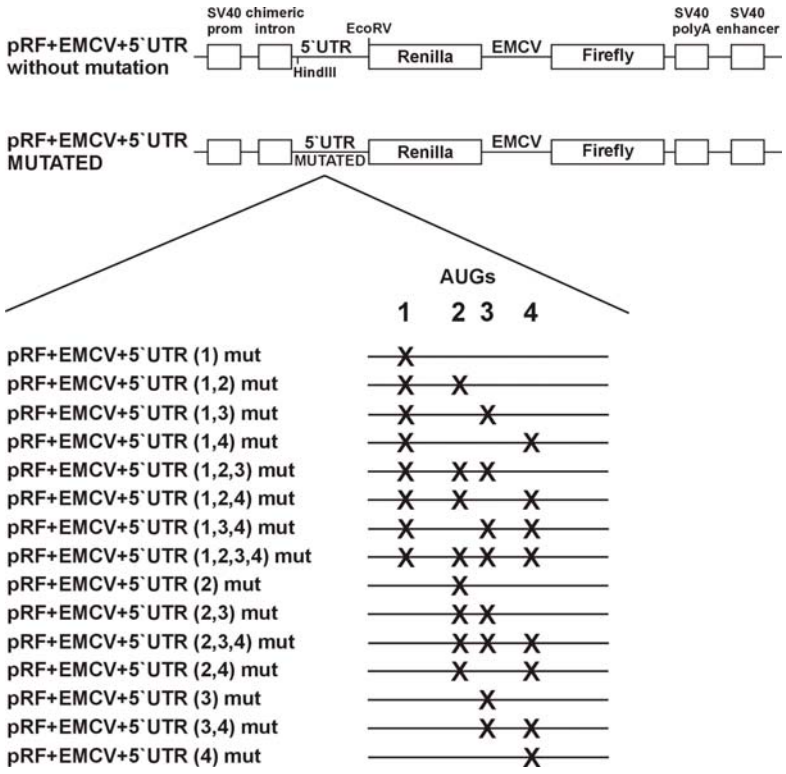
### 2.1.10.3. Promoter characterization

In order to map the cryptic promoter in the *myeov* 5`UTR, nested deletions of the *myeov* 5`UTR were generated. *Pfu* DNA polymerase was used to amplify the complete *myeov* 5`UTR using a cloned cDNA 11SMNp14m81 and the following primers: EcoRImyeov5UTRdel1for, EcoRImyeov5UTRdel2for, EcoRImyeov5UTRdel3for and myeov14NcoIUTRrev. A´s were added and the purified fragment was inserted into pGEM-T easy vector, creating pGEM-T+UTRdel1, pGEM-T+UTRdel2 and pGEM-T+UTRdel3. Correct insertion was checked by sequencing using M13for and M13rev primers. The fragments were digested with EcoRI and NcoI restriction endonucleases and the gel purified fragment was inserted into the pRF vector via EcoRI and NcoI sites, creating pRF+UTRdel1, pRF+UTRdel2 and pRF+UTRdel3.



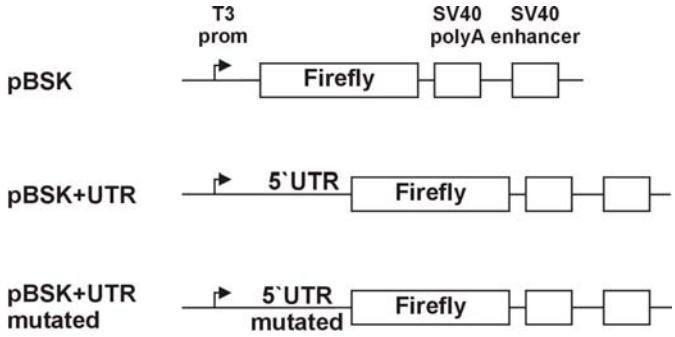
## 2.1.10.4. Characterization of the upstream open reading frame

To analyze the effect of the upstream open reading frame in the *myeov* 5`UTR on protein translation, *Pfu* DNA polymerase was used to amplify the complete *myeov* 5`UTR using a cloned cDNA 11SMNp14m81 as template and the primers myeovUTRHindIIIfor and myeovEcoRVrev. A´s were added to the PCR fragments with Taq polymerase, gel purified and inserted into pGEM-T easy vector, creating the construct pGEM-T+myeov5`UTRHindIII/EcoRV. Correct cloning was checked by sequencing using M13for and M13rev primers. In order to mutate the uAUGs within the *myeov* 5`UTR, pGEM-T+myeov5`UTRHindIII/EcoRV was used as template and the QuikChange®Multi Site-Directed Mutagenesis Kit (Stratagene) with the mutagenesis primers myeovATG1, myeovATG2, myeovATG3 and myeovATG4. Introduced mutations were verified by DNA sequencing using M13for and M13rev primers, and the inserts, myeov5`UTRmut (containing different mutation combination) as well the myeov 5`UTR wild type were subcloned into pRF+EMCV upstream of the Renilla luciferase cistron via HindIII and EcoRV sites. The cloned fragments were verified by DNA sequencing using a specific labelled Renilla luciferase reverse primer (RenillaLucRev).

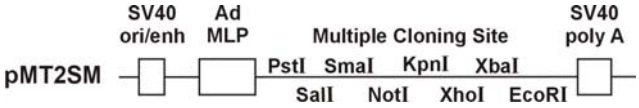


### 2.1.10.5. *In vitro* transcription for RNA transfection

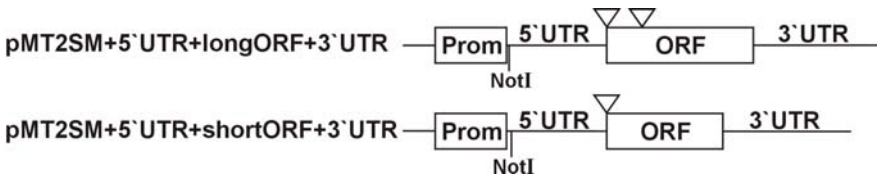
For in vitro transcription experiments using the T3 polymerase, we used a bluescript based plasmid (Stratagene) containing the SHOX 5`UTR upstream of the Firefly luciferase cistron with the SV40 polyA adenylation site and SV40 enhancer (Blaschke et al., 2003). For this purpose the SHOX 5`UTR was replaced by either the wild type or the mutated *myeov* 5`UTR using the SpeI/NcoI restriction sites, originating pBSK+UTR and pBSK+UTRmut$_{1,2,3,4}$ respectively.




### 2.1.10.6. Cloning of *myeov* cDNA fragments into the eukaryotic expression vector pMT2SM



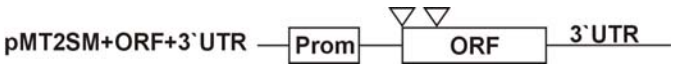

The complete *myeov* cDNAs encoding the long (11SMNp14m82) and the short ORF (11SMNp2m69) were digested with NotI from the lambda gt10 cloning vector and ligated into pMT2SM via NotI restriction site, thus creating pMT2SM+5`UTR+longORF+3`UTR and pMT2SM+5`UTR+shortORF+3`UTR, respectively.



To create the construct pMT2SM+5`UTRmut+ORF+3`UTR a double ligation was performed. For that, the *myeov* 5`UTR containing the 4 mutations in the uAUGs was obtained from pGEM-T+UTR$_{1,2,3,4mut}$ and digested with NotI and PflFI restriction endonucleases. The *myeov* cDNA encoding the long ORF together with the 3`UTR was obtained from the plasmid 11SMNp14m81 using the same restriction endonucleases and both fragments were ligated into the NotI site of pMT2SM.

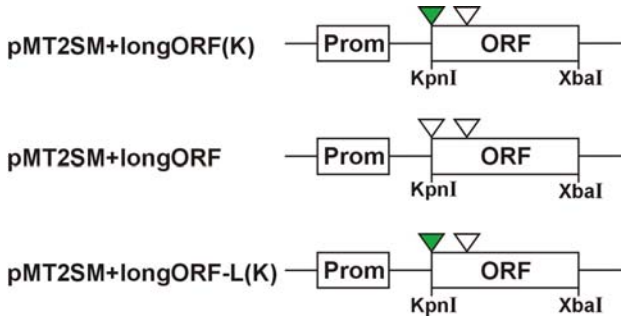

To generate a construct that lacks the 3`UTR (pMT2SM+5`UTR+ORF), the plasmid 11SMNp14m81 was digested with AatII, polished and subsequently digested with the restriction enzyme NotI. The purified fragment was ligated into the vector pMT2SM that was digested with EcoRI, polished and subsequently digested with NotI.


To construct the plasmid lacking the 5`UTR (pMT2SM+ORF+3`UTR) the plasmid 11SMNp14m81 was digested with PflFI, polished and then digested with the restriction enzyme NotI. The purified fragment was ligated into pMT2SM which was digested with PstI, polished and digested with NotI.

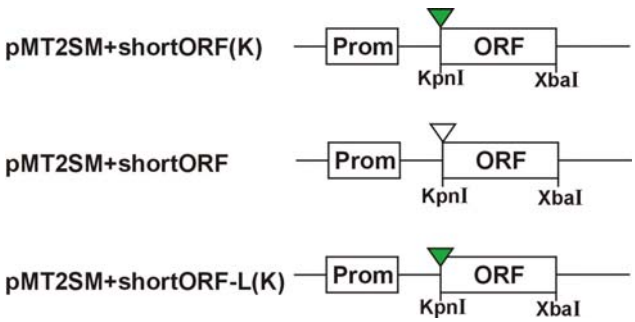



To create constructs of *myeov* ORF without 5` and 3`UTR sequences in the eukaryotic expression vector pMT2SM several amplifications were performed. The *myeov* encoding the long ORF containing the start codon in a perfect Kozak context was created by amplification using the plasmid 11SMNp14m81 as template and the primers myeov-Kpn-Kozak-long-14 and myeov-Xba-end, thus creating pMT2SM+longORF(K). To create a construct of

*myeov* long ORF containing its own ATG (suboptimal Kozak context), the same procedure was performed, except that the forward primer used was myeov-Kpn-own-long-14, creating in this way pMT2SM+longORF. In order to construct pMT2SM-longORF-L(K) the 11SMNp14m81 was used as template and the primers myeov-Kpn-Kozak-long-14 and myeov-Xba-minusL-end. This construct encodes the *myeov* long ORF without the leucine/isoleucin tail (Figure 3.1).



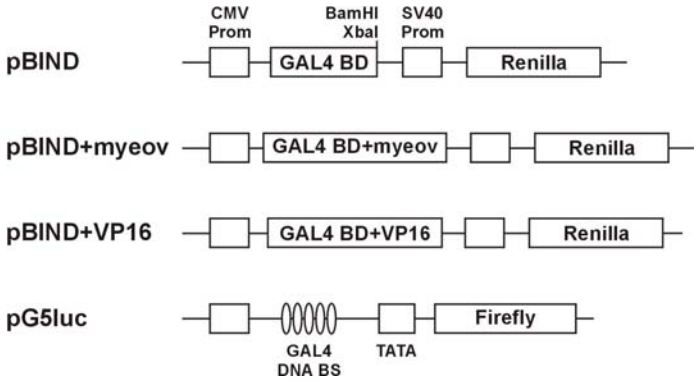

Constructs of *myeov* cDNA encoding the short ORF containing the start codon in a optimal Kozak context was created by amplification using the plasmid 11SMNP2m69 as template and the primers myeov-Kpn-Kozak-short-2 and myeov-Xba-L-end thus creating pMT2SM+shortORF(K). To create a construct of *myeov* short ORF containing its own ATG (suboptimal Kozak context), the same procedure was performed, except that the forward primer used was myeov-Kpn-own-2-short, creating in this way pMT2SM+shortORF. The construct pMT2SM-shortORF-L(K) was created using the same DNA template and the primers myeov-Kpn-Kozak-short-2 and myeov-Xba-end. This construct does not contain the leucine/isoleucin tail of *myeov* ORF (Figure 3.1).



All inserts were verified by sequencing using the pMT2SMfor and pMT2SMrev primers.

### 2.1.10.7. Transcription factor

To check whether *myeov* ORF codes for a transcription factor, the *myeov* long ORF was amplified with Pfu polymerase using the plasmid 11SMNp14m81 as template and the primers MyeovBampINDfor and myeovXbaEnde. A`s were added to the PCR fragment and inserted into pGEM-T easy vector, originating pGEM-T-myeovORFBamXbaI. The insert was checked by sequencing using M13for and M13rev primers. The purified fragment was subcloned in frame with Gal4-BD into the vector pBIND (Promega), thus creating pBIND+myeov. The same procedure was performed to create the construct pBIND+VP16, however, using the vector pACT as template and the primers BamVP16for and XbaVP16rev. All constructs were verified by sequencing using the specifically labelled Renilla luciferase primer (RenillaLucRev). The pG5luc was also purchased from Promega.



## 2.2. Methods

### 2.2.1. Cell Culture

All the human cell lines described here were maintained in a humidified atmosphere at 37°C containing 5% $CO_2$. The semiadherent cells were cultivated in 24 ml of medium using 175 $cm^2$ cell culture flasks (Greiner). Cells were splitted twice a week as follow: the cell monolayer was washed with prewarmed PBS. To detach the cells from the plastic surface, 2 ml of prewarmed trypsin/EDTA was added and incubated until the cells detached (usually 1-5 min depending on the cell line used). To stop trypsinization, medium with serum was added, and cells were cultured as described above. When necessary cells were counted using a hematocytometer and the numbers of required cells were transferred to a specific cell culture flask.

#### 2.2.1.1. HEK 293 cell line

Human fibroblast embryonic kidney cells (HEK-293) were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/HAM`S F-12 supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin.

#### 2.2.1.2. Met-5A cell line

Mesothelial cells (Met-5A) were isolated from pleural fluids obtained from non-cancerous individuals. This cell line stains positively for vimentin, keratins and SV40 T antigen. The SV40 large T antigen promotes replication from the SV40 origin, which is found in all luciferase plasmid vectors used in this study. The combination of large T antigen and SV40 origin may result in a higher copy number of these vectors, which in turn may result in increased expression of the reporter gene compared to other cell lines and vector combinations. Met-5A cells were kindly donated by Dr. Ling Chen (Heidelberg University Pediatric Hospital). The recommended medium for this cell line was Medium 199 with Earle's BSS, 75 mM L-glutamine and 1.25 g/L sodium

bicarbonate supplemented with 3.3 nM human epidermal growth factor (EGF), 400 nM hydrocortisone, 870 nM insulin, 20 mM HEPES and 10% FBS and 100 U/ml penicillin/streptomycin.

### 2.2.1.3. Capan-1 cell line

Capan-1 is a human pancreas adenocarcioma established from the liver metastasis of a pancreatic ductal adenocarcinoma from a 40-year-old Caucasian man in 1974. This cell line was described to grow in nude mice, induce metastases and was resistant to 5-FU (Fogh et al., 1977; Kyriazis et al., 1986). The cell medium recommended was RPMI 1640 + 20% FBS and 100 U/ml penicillin/streptomycin. Capan-1 was obtained from the DKFZ Tumorbank. Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH (DSMZ nº ACC 244).

### 2.2.1.4. Capan-2 cell line

Capan-2 is a human pancreas adenocarcinoma established from the tumor of a 56-year-old Caucasian man with pancreas adenocarcinoma in 1975 (Kyriazis et al., 1986). The recommended medium was RPMI 1640 + 15% FBS and 100 U/ml penicillin/streptomycin (DSMZ nº ACC 245).

### 2.2.1.5. DAN-G cell line

DAN-G is a human carcinoma cell line established from a human individual with pancreas carcinoma in 1985. The recommended medium was RPMI 1640 + 10% FBS and 100 U/ml penicillin/streptomycin (DSMZ nº 249).

## 2.2.2. Freezing of cells

The cells to be stored were briefly washed with prewarmed PBS, harvested by trypsinization and media containing serum was added to stop

the trypsin action. Cells were pelleted by centrifugation at 300 *g* for 5 min and resuspended in freezing media, aliquoted in 1 ml cryogenic vials and stored at – 80ºC overnight in a temperature controlled box (Mr. Frosty, Sigma). Next day the cells were transferred to liquid nitrogen.

| **Freezing media** | |
|---|---|
| DMEM | 70 % |
| DMSO | 10 % |
| FBS | 20 % |
| (autoclaved) | |

## 2.2.3. Thawing of cells

Thawing of stored cells was achieved by incubating the vials in a 37ºC water bath. Cells were removed from the vial and placed in a 15 ml sterile falcon tube to which slowly (dropwise) prewarmed medium was added. Cells were pelleted by centrifugation for 5 min at 300 x *g*. The cells were then transferred into a 75 $cm^2$ flask and cultivated by conventional methods. The next day cells were briefly washed with prewarmed PBS to remove dead cells, and fresh medium was added.

## 2.2.4. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) designed by Karry Mullis (Mullis et al., 1986) has been and is extensively used in molecular biological research, medical diagnostics and forensic sciences. This technique is used to amplify exponentially a specific DNA sequence. For a PCR reaction the following ingredients are necessary: DNA template, buffer, oligonucleotide primers, dNTPs (mix containing the four deoxyribonucleoside triphosphates), and a heat stable DNA polymerase. The PCR reaction comprises different temperature cycles which correspond to basically three steps:

1. **DNA Denaturation.** The DNA strands are separated by setting the temperature at 95°C for 15 seconds, forming two single DNA strands that allow hybridization with oligonucleotide primers.
2. **Annealing of primers.** The temperature is lowered to 56°C to allow the primers to hybridize to the target sequence. The primers are designed in such a way that one primer hybridizes to the 5`-end and the other primer to the complementary strand at the 3`-end of the target sequence. Since there is excess of primers, the parental DNA does not reanneal.
3. **DNA synthesis.** The temperature is raised to 72°C, which is the optimal temperature for a Taq DNA polymerase to synthesize target DNA. Starting from the 5`- and 3`- primer, a copy of both strands of parental DNA is synthesized.

By repeating these cycles, the target DNA between the two primers can be exponentially amplified. The PCR reaction was pipetted using filter tips and was performed in a clean area, the following components were pipetted into a sterile PCR tube.

| **PCR reaction** | | |
|---|---|---|
| Template DNA | ~100 | ng |
| Oligonucleotide primer (each) | 0.3 | pmol |
| dNTPs | 0.2 | mM |
| Buffer | 1 | x |
| Magnesium | 0.2 | mM |
| DNA Polymerase | 2.5 | U |
| Water to | 100 | µl |

The enzyme *Pfu* polymerase was used preferable because this enzyme has a 3`→ 5`exonuclease (proofreading) activity and thus a lower error rate compared to other thermostable polymerases (Lundberg et al., 1991). As control, an additional reaction was performed without template DNA. The protocol described above is a standard protocol. However, the melting temperature (Tm) was calculated individually for each set of primers used. To

calculate the optimal melting temperature, the following formula has been used:

**Tm = 69.3ºC + 0.41x (GC-Percent) – 650/Primer Length**

The annealing temperature was mostly set 3-5ºC bellow the calculated melting temperature.

## 2.2.5. PCR Polishing

The PCR Polishing Kit (Stratagene) was used to polish the ends of 3`-overhang extensions of polymerase-generated DNA fragments or to fill-in 5` overhangs to generate blunt-ends. The following components were added into a sterile 0.5 ml microcentrifuge tube:

| **Polishing reaction** | | |
|---|---|---|
| Digested DNA or PCR product | ~500 | ng |
| 10 x *Pfu* buffer (containing magnesium) | 2 | µl |
| 2.5 M dNTP | 2 | µl |
| *Pfu* polymerase | 5 | U |
| Water to | 20 | µl |

The contents were gently mixed and incubated in a thermal cycle at 72ºC for 30 minutes. The mixture was phenol-chlorophorm-isoamylalchohol extracted and ethanol precipitated (section 2.2.16 and 2.2.17, respectively).

## 2.2.6. A-tailing reaction

PCR products obtained with *Pfu* polymerase do not contain an adenosine nucleotide at its 3`- terminal and therefore are not compatible for direct ligation into pGEM®-T easy vector (Promega), which contains a 3`-terminal thymidine at both ends. However, an A-tailing reaction can be

performed to add adenosine nucleotides to the PCR fragment ends, making it possible to ligate the fragment into the pGEM-T easy vector.

To perform the A-tailing reaction for blunt-ended DNA or PCR fragments generated by *Pfu* Polymerase, the DNA was ethanol precipitated and resuspended in 20 µl water. The contents described below were mixed into a sterile PCR tube and incubated in a thermal block for 30 min at 70°C.

| **A-tailing procedure** | | |
|---|---|---|
| Purified PCR fragment | 10 | µl |
| 10 x Buffer | 5 | µl |
| $MgCl_2$ (end concentration) | 2 | mM |
| dATP (end concentration) | 0.2 | mM |
| Taq DNA polymerase | 1 | U |
| Water to | 50 | µl |

## 2.2.7. Plasmid DNA transformation

To introduce plasmid DNA or recombinant plasmid DNA into bacteria, the bacteria had to be made competent for this purpose. We used competent cells purchased from Invitrogen and Stratagene or we homemade competent cells according to the following protocols.

### 2.2.7.1. Preparation of chemically competent cells – calcium chloride method

Bacteria were made competent for transformation according to a protocol provided by Sambrook et al (Sambrook et al., 1989). Working aseptically, DH10B cells (taken from a glycerol stock culture) were streaked out on a LB plate and incubated overnight at 37°C. The next day, one bacterial colony (2-3 mm in diameter) was picked and inoculated into 100 ml of LB medium in a 1-liter erlenmeyer, and the solution was incubated at 37°C in a rotatory shaker. Cell density was measured by a spectrophotometer at

$OD_{600}$. When an $OD_{600}$ of 0.18 to 0.22 was reached, the bacteria were transferred to a Falcon tube 2059 and incubated on ice for 10 min.

The cells were spun down at 2,700 *g* for 10 min at 4°C, the supernatant was discarded and the bacterial pellet was resuspended in 10 ml ice-cold 0.1 M $CaCl_2$ (from a 1M stock solution, the solution was filter sterilized through a filter of 0.45 µm pore size) and incubated on ice for 20 min. Centrifugation was performed again, and the cells were resuspended in 3.410 µl of 0.1 M $CaCl_2$ plus 590 µl of 87% glycerol. The cells were used immediately for transformation and/or distributed into several prechilled sterile microfuge tubes. The competent cells were frozen immediately by immersion of the microfuge tubes in liquid nitrogen and stored at – 80°C in 50 µl aliquots.

## 2.2.7.2. Preparation of electrocompetent cells - GYT method

To prepare electrocompetent cells using the GYT method (Tung and Chow, 1995), a single bacteria colony was picked from a LB-agar plate and inoculated into 3 ml LB media and grown overnight in a shaker at 37°C. The next day, the culture was diluted in 300 ml LB medium and incubated at 37°C with vigorous shaking until the $OD_{600}$ reached 0.6. The cells were then incubated on ice for 30 minutes and harvested at 4000 *g* for 15 min at 4°C. The bacteria were washed twice with 25 ml ice-cold 10% glycerol and resuspended in a final volume of 0.6 ml ice-cold GYT. The cells were used immediately for transformation and/or distributed into several prechilled sterile microfuge tubes. Working quickly the competents cells were frozen immediately by immersion of the microfuge tubes in liquid nitrogen and the cells were stored at – 80°C in 50 µl aliquots.

| **GYT** | | |
|---|---|---|
| Glycerol | 10 | % |
| Yeast extract | 0.125 | % |
| Tryptone | 0.25 | % |

## 2.2.8. Transformation of competent cells

Transformation is the term used to describe the introduction of plasmid DNA into bacteria. The experiment can be performed by an electrical or a chemical method. The electrical method, called electroporation (Andreason and Evans, 1988; Shigekawa and Dower, 1988; Tur-Kaspa et al., 1986) is a method which uses electric field pulses of high voltage (kV/cm) and short duration (microsecond range) to introduce foreign DNA into mammalian cells or bacteria. Electroporation is temperature-dependent and is best carried out at 0-4°C. The efficiency of transformation drops as much as 100-fold when electroporation is carried out at room temperature (Sambrook and Russell, 2001).

The chemical method on its turn consists of a heat shock to introduce the DNA into the host. Routinely we used electroporation, but for some experiments the chemical method was applied.

### 2.2.8.1. Electroporation

In order to transform bacteria by electroporation we either used homemade electrocompetent cells (section 2.2.7.2) or the competent TOP10 cells purchased from Invitrogen. The TOP10 cells were chosen because these cells have high transformation efficiency of 1 x $10^9$ cfu/μg supercoiled DNA and contain the genotype ϕ80lacZΔM15 which allows blue/white color screening of recombinant clones. The electrocompetent cells were stored at -80°C and were thawed on ice, and 20 μl of the cells were diluted once with sterile water and mixed with 1 to 2 μl (~25 ng DNA) of the ligation reaction (or 10 ng of plasmid DNA) in a prechilled eppendorf tube and incubated for 1 min. on ice. The reaction was transferred to a prechilled sterile cuvette (1 mm) and electroporated with an electrical pulse of 25 μF capacitance, 1.8 kV and 200 ohm resistance. Immediately after electroporation 1 ml of pre-warmed LB media (without antibiotics) was added to the cells, followed by incubation for 1 hour at 37°C with gentle rotation to allow cells to recover. Up to 200 μl of the transformation reaction was plated onto 90-mm LB agar plates (containing

the required antibiotic according to the antibiotic resistance gene cassette presented on the plasmid DNA). The LB-agar plates were left open for a few minutes under the fume hood (sterile) until the liquid was absorbed, the plates were closed and incubated inverted overnight at 37°C.

### 2.2.8.2. Chemical transformation

For transformation of mutated plasmid DNAs (section 2.2.21) we used the chemical transformation method as recommended by the manufacturer (Stratagene). XL2-Blue or XL10-Gold ultracompetent cells were thawed on ice, and 45 µl was placed in a prechilled falcon 2059 polypropylene tube, and 2 µl β-ME was added to the cells. The contents of the tube were swirled gently and incubated on ice for 10 minutes, every two minutes the tube was swirled, and 1.5 µl of the Dpn I-treated DNA (see section 2.2.21) was transferred to the tube containing the ultracompetent cells. The transformation reaction was swirled and incubated on ice for 30 minutes. The tube was heat-pulsed in a 42°C water bath for 30 seconds and incubated on ice for 2 minutes. 900 µl of preheated (42°C) $NZY^+$ broth media was added and incubated at 37°C for 1 hour in a shaker at 225-250 rpm. Different volumes were plated onto LB-agar plates (containing the antibiotic for selection) and incubated inverted overnight at 37°C.

## 2.2.9. Plasmid Preparation

### 2.2.9.1. Small scale plasmid DNA preparation (mini-prep)

Plasmid mini preparation was performed basically as described by Sheibani and Frazier, with small modifications (Sheibani and Frazier, 1997). A single bacterial colony was picked with a sterile inoculating loop and inoculated into 5 ml LB media (containing the required antibiotic for selection) into a 15 ml falcon tube, and grown overnight with vigorous shaking at 37°C.

The following day, 1.5 ml of the culture was transferred to an eppendorf tube, and the bacteria were recovered by centrifugation for 3 min at 10,000 *g*. Supernatant was removed and a second 1.5 ml bacterial culture was added and centrifuged. The supernatant was again discarded (the rest (2 ml) of the culture was kept at 4°C). The bacterial pellet was resuspended by vortexing, and 300 µl ice-cold buffer P1 (Table 2.2) was added to the cells and mixed by inverting the tube 10 times. To lyse the cells, 300 µl (RT) buffer P2 was added, mixed by inverting the tube 6 times and incubated at RT for up to 5 minutes. Lysis was stopped by addition of 300 µl ice-cold buffer P3. The mixture was centrifuged for 30 min at 10,000 x *g* and the supernatant was transferred to a new eppendorf tube. To precipitate the DNA, 600 µl isopropanol was added, and the DNA pellet was obtained by centrifugation for 30 min at 10,000 x *g*. The supernatant was discarded and the DNA pellet was washed with 500 µl 70% ethanol. Finally DNA was dried at 65°C and dissolved in 30 µl 10T 0.1E buffer for 5 min at 65°C at 800 rpm.

**Table 2.2. Buffers used for plasmid DNA mini preparation**

| **Buffer P1** | **Buffer P2** | **Buffer P3** |
|---|---|---|
| 50 mM Tris/HCl, pH 8.0 | 200 mM NaOH | 3 M K acetate, pH 5.5 |
| 10 mM EDTA | 1% SDS | |
| 100 µg/ml Ribonuclease A* | mix before use | |
| (Storage at 4°C) | (Storage at RT) | (Storage at RT) |

* Ribonuclease A was made with 10 mg/ml RNase A, 15 mM NaCl and 10 mM Tris/HCl, pH 7.5. The solution was incubated at 90°C for 30 min, cooled to room temperature and stored at -20°C.

## 2.2.9.2. Large scalle plasmid DNA preparation (maxi-prep)

### 2.2.9.2.1. Ammonium Acetate Method

To obtain plasmid DNA on a large scale, we used the ammonium acetate protocol. The advantage of this method is the amount of DNA obtained compared to commercially available kits, e.g. the maximum yield using the QIAGEN®-tip 500 Column ranged from 250 to 600 µg of DNA per 100

ml culture while the ammonium acetate protocol yielded between from 750 to 5000 µg of DNA per 100 mL culture using high-copy-number plasmids. Besides this, the cost of this method is much less compared to that of commercial kits. The protocol described is from Saporiton-Irwin et al with small modifications (Saporito-Irwin et al., 1997).

Plasmid DNA was usually prepared as follows: 50 µl mini-prep culture was inoculated in 500 ml LB media (containing the respective antibiotic for selection) in a 2 liter erlenmeyer, and grown overnight at 37°C with vigorous shaking.

The next day, the bacterial pellet was recovered by centrifugation in 250 ml beakers (all the centrifugation steps were performed at 10,000 x *g* at 4°C, except as noted) and the bacterial pellet was resuspended with vigorously vortexing in 12 ml of freshly prepared Solution 1 (Table 2.3) and incubated at 37°C for 30 min at 100 rpm to ensure even distribution. After incubation the cells were kept on ice for 20 min.

To lyse the cells, 24 ml of freshly made Solution 2 (Table 2.3) was added and mixed by inverting the tube and directly incubated on ice for 10 min. Next, 18 ml ice-cold 7.5 M ammonium acetate (pH 7.6) was added and mixed by inversion and again incubated on ice for 10 min. Centrifugation was accomplished for 30 min. After centrifugation the solution was filtrated and 27 ml of isopropanol was added followed by incubation for 10 min at RT. The solution was centrifuged once more for 10 min and the pellet containing the plasmid DNA was resuspended in 4 ml 2 M ammonium acetate (pH 7.4) and transferred to a falcon 2059 polypropylene tube and incubated either for 10 min on ice or at 4°C overnight. Next, the sample was centrifuged for 10 min, and the supernatant was placed into a new falcon 2059 polypropylene tube, and 4 ml isopropanol was added and mixed by inverting the tube. The sample was incubated for 10 min at RT and centrifuged for 10 min. The pellet obtained after centrifugation was resuspended in 2 ml distilled water and 10 µl (5 mg/ml) RNase was added followed by incubation for 30 min at 37°C. Optionally extraction with phenol:chloroform:isoamylalcohol was performed.

Following RNase treatment, 1 ml of ice-cold ammonium acetate (pH 7.6) was added, mixed by inversion and incubated for 5 minutes at RT, followed by a centrifugation step for 10 min. The supernatant was transferred to a falcon 2059 tube and 3 ml isopropanol was added and mixed by inverting the tube and incubated for 10 min at RT, followed by a centrifugation for 10 min at RT. Finally the obtained DNA pellet was washed with 70% ethanol, dried at 65°C and resuspended in 300 – 500 µl TE buffer.

**Table 2.3. Buffers used for plasmid DNA maxi preparation**

| Solution 1 | Solution 2 |
|---|---|
| 25 mM Tris/HCl, pH 7.6 | 1% SDS |
| 10 mM EDTA pH 8.0 | 200 mM NaOH |
| 50 mM Glucose | |
| 24 mg hen egg lysozyme | |

## 2.2.9.2.2. Maxi-prep (Qiagen)

The DNA Maxi preparation was performed using the Qiafilter Plasmid Maxi Prep Kit according to the suppliers protocols (Qiagen® Plasmid Handbook). The solutions used are shown in Table 2.4. Centrifugation steps were performed at ≥ 10,000 x *g* or, except as noted.

A single colony was picked from a LB plate and inoculated into 5 ml LB medium (containing the required antibiotic). The cells were grown for ~8 hours (late logarithmic phase), and this miniculture was diluted 1:100 into 500 ml LB/Amp media and grown to saturation overnight at 37°C under vigorous shaking.

The next day bacteria were harvested by centrifugation at 3,800 x *g* for 15 min at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 10 ml of buffer P1.

To lyse the cells, 10 ml of buffer P2 was added and the samples were gently mixed by inverting the tube 4-6 times and then incubated at room

temperature for 5 min. To stop lysis, 10 ml prechilled buffer P3 was added and the samples were mixed by inverting the tube 4-6 times, followed by an incubation on ice for 20 min.

Samples were mixed again, and the clear supernatant obtained after centrifugation for 30 min at 4°C was transferred to a new tube. The tube was recentrifuged for 15 min, and the supernatant was transferred to a new tube.

The QIAGEN-tip 500 columns were equilibrated by applying 10 ml of buffer QBT, and the column was allowed to drain by gravity flow. The supernatant was applied to the column and allowed to enter the resin by gravity flow. The column was washed 2 times with 30 ml of buffer QC.

For elution, 15 ml of buffer QF was added. DNA in the collected eluate was precipitated by adding 0.7 volumes (10.5 ml) of isopropanol, followed by centrifugation at 4°C for 30 min.

The DNA pellet was briefly washed with 5 ml of ice-cold 70% ethanol and again centrifuged for 15 min at 4°C. DNA pellet was dried at 65°C and then resuspended in 200-500 µl of sterile water or TE buffer.

**Table 2.4. Solutions for maxi preparation**

| **P1** | **P2** | **P3** |
|---|---|---|
| 50 mM Glucose | 0.2 M NaOH | 3 M Potassium acetate |
| 25 mM Tris-HCl, pH 8.0 | 1% SDS | 2 M Acetic acid |
| 10 mM EDTA, pH 8.0 | | |
| **QBT** | **QC** | **QF** |
| 750 mM NaCl | 1 M NaCl | 1.25 M NaCl |
| 50 mM MOPS | 50 mM MOPS | 50 mM Tris |

## 2.2.10. Determination of nucleic acid concentration

Recovery, purity and concentration of nucleic acids were determined by spectrophotometric analysis. The ratio of absorbance at ($A_{260}$-$A_{320}$/$A_{280}$-$A_{320}$) should be 1.8 to 2.0 for DNA and higher as 2.0 for RNA. In presence of protein contamination the ratio is less. For the measurement we used a

spectrophotometer from Pharmacia Biotech. The DNA or RNA was diluted 1:100 in distilled water and transferred to a quartz cuvette. The absorption was at wavelengths of 260 nm, 280 nm and 320 nm. An optical density (OD) of 260 nm of 1.0 is equivalent to 50 µg/ml DNA, 40 µg/ml RNA or 20 µg/ml oligonucleotides. The spectrophotometer machine used provides us with the concentration and the ratio automatically. Nevertheless, the formula used to calculate the concentration (C) and molarity (M) were the following:

**C = OD 260nm x dilution factor x equivalent = x µg/ml**

**M = Concentration (µg/ml) x 1,000/330 = x pmol/µl**

## 2.2.11. DNA cleavage with restriction endonucleases

Restriction enzymes, also called restriction endonucleases are bacterial proteins which work as an immune system in bacteria. Their role is to destroy bacteriophages or other viruses which invade bacteria. Restriction endonucleases recognize a specific nucleotide sequence, and cut DNA wherever this specific sequence is found. Usually the palindromic restriction sites have a length of 4 to 8 base pairs. The purified restriction endonucleases are commercially available, and are predominantly used to generate DNA fragments for cloning experiments or Southern blot analysis. Therefore, restriction endonucleases are a major tool in recombinant DNA technology.

Normally DNA was cleaved at 37°C for 2 hours in a total volume reaction of 100 µl with 1 or 2 X buffer (supplied by the manufacturer) and 5-fold enzyme units per microgram DNA. The enzyme Sma I was an exception and was incubated at 25°C overnight.

## 2.2.12. Dephosphorylation

Calf intestinal phosphatase (CIP) or shrimp alkaline phosphatase (SAP) (Seeburg et al., 1977; Ullrich et al., 1977) are enzymes which can remove the DNA 5` phosphate group, and therefore suppress self-ligation and

recircularization of the plasmid DNA used for cloning. In this way the ligation (fragment-plasmid) efficiency is enhanced and background of transformed bacterial colonies that carry "empty" plasmids (plasmid without insert) is reduced.

Desphophorylation was performed according to the protocol of Sambrook and Russel with small modification (Sambrook and Russell, 2001). Digested plasmid DNA was phenolized, ethanol precipitated and resuspended in 89 µl distilled water, and then the complete reaction was performed in three incubation steps:

1) The 89 µl DNA solution was incubated for 30 min at 37°C with 10 µl CIP-buffer (10 x) and 1 µl CIP (18 U);

2) An additional microliter of CIP was added and incubation was proceeded for 30 min at 56°C;

3) The desphosphorylation reaction was stopped by adding 4 µl of 0.5 M EDTA and incubation for 10 min at 68°C.

## 2.2.13. Ligation

T4 DNA ligase is encoded by the gene 30 of bacteriophage T4 (Wilson and Murray, 1979). This enzyme can be used to ligate DNA restriction fragments. T4 DNA ligase has the capacity to catalyze *in vitro* the formation of a phosphodiester bond between adjacent nucleotides, one containing a terminal 5-phosphate group and one containing the hydroxyl terminus. A review of the ligation reaction can be seen in (Cherepanov and de Vries, 2003; Madrid et al., 1998; Shuman, 1996).

In my study, three types of ligation were performed: cohesive, blunt-end and recircularization, and they are briefly discussed below and schematically shown in table 2.5.

### 2.2.13.1. Ligation of cohesive ends

Mostly restriction endonucleases create cohesive ends. When fragment DNA and plasmid DNA are digested with the same restriction endonuclease, complementary cohesive ends are generated that can easily be ligated. For such kind of ligation, incubation at 4 or 16ºC overnight was performed. Optionally incubation at room temperature was also performed; however, ligation rates are lower when compared to overnight incubation.

**Table 2.5. Ligation reactions and pipetting scheme**

| Contents | Cohesive end | Blunt-end | Recircularization |
|---|---|---|---|
| Plasmid DNA | ~ 50 ng | ~ 200 ng | ~ 50 ng |
| Insert DNA | ~ 150 ng | ~ 600 ng | - |
| 50% PEG 4000 | - | 2 µl | 5 µl |
| 10 x buffer | 1 µl | 2 µl | 5 µl |
| Ligase | 1 U | 5 U | 5 U |
| Total volume reaction | 10 µl | 20 µl | 50 µl |
| Incubation time | 4ºC or 16ºC overnight | 22ºC for 1 hour | 22ºC for 1 hour |
| Phenolization and ethanol precipitation | no | yes | no |

### 2.2.13.2. Blunt-end ligation

PCR products originated from *Pfu* DNA polymerase are blunt-end. Some enzymes, e.g. Sma I also makes blunt end DNA. In contrast to cohesive ends, blunt-ends are difficult to ligate, and require high concentration of DNA and T4 DNA ligase. Blunt-end ligation is stimulated when using low amounts of polyethylene glycol (Rusche and Howard-Flanders, 1985; Zimmerman and Pheiffer, 1983), therefore, the blunt-end ligation was performed in the presence of PEG at a temperature of 22ºC for 1 hour. The enzyme was inactivated at 65ºC for 10 min, and the ligation reaction was phenolyzed and ethanol precipitated (section 2.2.16 and 2.2.17, respectively).

### 2.2.13.3. Recircularization

In some cases, it was only necessary to recirculize the plasmid DNA. For example when producing promoterless constructs, the promoter region was removed by restriction enzyme digestion, and the linearized gel purified plasmid DNA was religated. For this purpose, a large reaction volume was used to enhance the chance that plasmid DNA would self-religate in stead of ligation to an adjacent linearized plasmid DNA fragment.

## 2.2.14. Agarose gel electrophoresis

Agarose is a linear polymer composed of residues of D- and L-galactose and is used to separate fragments of DNA or RNA by size (Sambrook and Russell, 2001). Since deoxyribonucleic acids are negatively charged, they migrate through the agarose gel in a electrical field toward the positive anode. Molecules of double-stranded DNA migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs, therefore small molecules migrate faster than large ones (Helling et al., 1974). There is a linear relationship between the logarithm of the electrophoretic mobility of the DNA and the gel concentration (Bearden, 1979; Calladine et al., 1991; Sambrook and Russell, 2001).

Plasmid DNA or DNA fragments obtained after treatment with restriction enzymes were separated by electrophoresis through an agarose gel. The agarose concentration used varied from 0.6% to 2.0%. The RNA electrophoresis is discussed separately in section 2.2.25.2.

### 2.2.14.1. Gel preparation

To prepare an agarose gel, the required amount of agarose was dissolved in 50 or 100 ml (small and big gel, respectively) 1 x running buffer (Table 2.6). The agarose was solubilized in a microwave oven until the agarose was completely dissolved (during boiling the water volume decreases by evaporation, therefore the water volume was replenished). The

gel was cooled to ≤ 45ºC and ethidium bromide was added to an end concentration of 0.5 µg/ml and mixed through gentle swirling. The agarose gel was then poured into a horizontal gel tray, and a comb for forming the sample slots was placed into the gel. The gel was solidified for about 30 min and then placed into an electrophoresis tank, where the gel was covered by the same batch of running buffer used to make the gel.

### 2.2.14.2. Sample preparation and running conditions

The DNA was mixed with 1/5$^{th}$ of loading buffer (Table 2.6) and the sample was placed into a well on the agarose gel. As fragment size control, a DNA standard Gene Ruler™ with either 100 bp or 1 Kb was used.

Electrophoretic separation was achieved by constant current at 5 V/gel cm for 60 to 90 minutes.

**Table 2.6. Runnning and loading buffer used for DNA electrophoresis**

| 1 x TBE | 1 x TAE (modified)* | Loading buffer |
|---|---|---|
| 90 mM Tris | 40 mM Tris-acetate, pH 8.0 | 10 mM Tris/HCl, pH 7.5 |
| 90 mM Boric acid | 0.1 mM $Na_2EDTA$ | 20% Ficoll |
| 2 mM EDTA | | 1 mg/ml Orange G |

***** Modified TAE has 0.1 mM $Na_2EDTA$ while standard TAE has 1.0 mM. The lower concentration of EDTA in the modified TAE does not interfere with the magnesium concentration required for downstream enzymatic reaction (Montage).

### 2.2.14.3. DNA visualization

DNA within agarose gels is only visible when stained with ethidium bromide and can then be visualized under UV light. The gel was placed onto an UV ilumuminator that emits UV light at 302 nm and photographed with a CCD camera connected to a computer (Epi Chemi II). Image files were saved, printed and subsequently analyzed. The size of the DNA was determined by comparing their mobility with the fragments of the Gene Ruler.

## 2.2.15. Isolation of DNA fragments from agarose

Isolation of DNA fragment from agarose gels was performed using the DNA Gel Extraction Kit or QIAquick Gel Extraction Kit as recommended by the manufacturers Montage and Qiagen, respectively:

### 2.2.15.1. Montage DNA Gel extraction kit

This kit contains a centrifugal filter device created to extract DNA fragments that range in size from 100 to 10,000 bp. When using this kit, the agarose gel and electrophoresis buffer was made with 1 x modified TAE buffer (Table 2.6). The agarose gel was placed above an UV table (Foto Prep I) and using a scalpel, a slice containing the desired DNA band was cut out from the agarose gel and placed into the Gel Nebulizer provided by the respective company. The Gel Nebulizer was connected to a sample filter cup, which was placed into a vial and centrifuged for 10 minutes at 5,000 x *g*. The centrifugation forces the agarose though the Gel Nebulizer, converting it into fine slurry that is captured by the Sample Filter Cup. The DNA passes through the microporous membrane in the sample filter cup and is collected in the vial.

### 2.2.15.2. QIAquick® Gel Extraction Kit

A slice containing the desired DNA band was excised from the gel. The gel slice was placed in a 1.5 ml eppendorf tube, and weighed. Three volumes of buffer QX1 were added to one volume of gel (e.g., 300 µl of buffer QX1 to each 100 mg of gel).

The gel slice was incubated at 50°C for 10 min to dissolve the agarose. To help gel dissolution the tubes were vortexed every 2 minutes. A QIAquick spin column was placed in a 2 ml collection tube. After the gel slice was dissolved completely, the solution was applied to the QIAquick column. This column was centrifuged for 1 min at 10,000 *g*. The DNA was bound to the column and the flow-through was discarded.

For washing, 750 µl of buffer PE was added to the column and centrifugation was performed for 1 min. The flow-through was discarded and centrifugation was repeated for an additional minute.

The column was placed into a 1.5 ml eppendorf tube, and elution of DNA was performed by adding 50 µl of water or TE buffer to the center of the column and incubated for 1 min. Subsequently eppendorf tube with column was centrifuged for 1 min. This step was repeated once to remove residual DNA from the column.

## 2.2.16. Phenol-chloroform extraction

An equal volume of phenol/chloroform/isoamylalcohol (PCI) was added to a DNA solution in a 1.5 ml eppendorf tube and vigorously vortexed. Subsequently the solution was centrifuged at 10,000 *g* for 3 min at RT, and the aqueous upper phase was transferred to a new eppendorf tube for ethanol precipitation.

## 2.2.17. Ethanol precipitation

One tenth volume of 3 M sodium acetate (pH 5-7) and 2 ½ volumes of 100% ethanol were added to the DNA solution and mixed. When having low DNA amounts, 1 µl glycogen (20 µg) was added additionally, followed by an incubation for 30 min at -80°C or 2 hours at – 20°C, and centrifugation for 30 min at 10,000 x *g*. The supernatant was carefully removed, and the DNA pellet was washed with 1 volume of room-temperature 70% ethanol, followed by centrifugation at full speed for 10 min, the supernatant was carefully removed, and the DNA pellet was dried at 65°C and dissolved into 10T 0.1E buffer at 65°C for 5 min.

## 2.2.18. Screening transformants for inserts by blue/white selection

Successful ligation of fragments cloned into the pGEM-T easy vector can be checked by color screening on indicator LB-agar plates. When a fragment is cloned into the pGEM-T easy vector, the fragment interrupts the coding sequence of the β-galactosidase gene and in turn the bacterial colonies will not turn blue. Therefore white colonies are picked for mini-prep and checked by restriction endonucleases.

Thirty minutes prior to plating the cells, 100 µl IPTG (100 mM), and 12.5 µl X-gal (80 mg/ml) were plated and incubated to allow the plates to absorb the liquid. The bacterial culture was plated onto a 90 cm plate and left open inside the fume hood until all the liquid was absorbed, then closed and incubated inverted at 37°C overnight.

## 2.2.19. DNA Sequencing

The sequencing method is based on the use of modified nucleotides, so called 2`, 3`-dideoxyl analogs (Sanger et al., 1977). These special nucleotides lack a hydroxyl residue at the 3`position of the deoxyribose. When DNA polymerase adds nucleotides into a DNA chain through its 5` triphosphate groups, the absence of a hydroxyl group on a dideoxyl analog avoids the formation of a phosphodiester bond with an adjacent nucleotide, leading to a stop in elongation. As the concentration of the analogs is very low, termination happens just occasionally. With four different analogous nucleotides in four separate reactions, numerous fragments corresponding to every base position will be synthesized. As fluorescently primers were used, newly synthesized DNA fragments are marked and the sequence can be detected by excitation with a laser and detection with photodiodes in a sequencing machine. We used the Li-cor machine, which has a dual laser, allowing the use of two labeled primers in a single tube reaction.

### 2.2.19.1. Gel preparation

Before gel preparation, the sequence plates (66 cm) were rinsed twice with distilled water and 80% ethanol, and dried with a precision wipe tissue. Glass plates were put together with spacer between them and fixed with the rails. The 66 cm gel was made by adding the compounds depicted below into 100 ml beaker:

| **Sequence gel** | | |
|---|---|---|
| Urea | 25.2 | g |
| 50% Acrylamide | 5.6 | ml |
| 10 x TBE | 6.0 | ml |
| Distilled water, complete to | 60.0 | ml |

The contents were mixed thoroughly and filtered through a 0.45 µm pore size filter. To polymerize the gel, 400 µl of 10% ammonium persulfate (APS) and 40 µl of N,N,N,N-Tetramethylethylendiamin (TEMED) were added to the solution, briefly mixed and injected between the plates.

### 2.2.19.2. DNA sequence reaction

The sequence reaction was prepared in a sterile PCR tube using a Cycle Reader™Auto DNA sequencing Kit (MBI Fermentas), which provides the nucleotides, enzyme and buffer. The nucleotide mixes were thawed on ice and 2 µl of each nucleotide mix (A, C, G and T) was added separately in each PCR tube. The following compounds were added in a eppendorf tube:

| **Reaction sequence** | | |
|---|---|---|
| DNA | ± 400 | ng |
| Buffer | 2.5 | µl |
| Each label primer | 2.0 | pmol |
| Polymerase | 2.0 | µl |
| Water to | 17 | µl |

The compounds were mixed and briefly centrifuged and 4 µl of the reaction was added in each PCR tube containing the nucleotide mixes. The

sequence reactions were performed using a thermal cycler with the following program:

| Segment | Cycles | Temperature | Time |
|---|---|---|---|
| 1 | 1 | 94°C | 3 minutes |
| 2 | 40 | 94°C | 30 seconds |
| | | 56°C | 30 seconds |
| | | 72°C | 1 minutes |

After termination of the program, 3 µl stop solution was added, mixed and briefly centrifuged.

### 2.2.19.3. DNA sequence electrophoresis

Electrophoresis was performed using a 4200 and 4300 DNA Analyzer (Li-cor) machine, which was connected to a computer. As running buffer, 0.8 x TBE buffer was used. Prior to sequencing, samples were heated to 90°C for 3 minutes and placed on ice, 1.5 µl was loaded and the gel was running for about 10 hours. The following day the results were analyzed with the DNA Sequencing and Analysis Software, version 3.0 (Li-cor).

| **Running conditions** | | |
|---|---|---|
| Voltage | 3000 | V |
| Current | 30 | mA |
| Power | 75 | W |
| Temperature | 45 | °C |
| Signal Filter | 1 | |
| Scann speed | 3 | |
| Frames | 30 | |

| **10 x TBE buffer** | | |
|---|---|---|
| Tris Base | 107.8 | g |
| Boric Acid | 55.8 | g |
| EDTA | 7.4 | g |
| Water to | 1 | L |

### 2.2.20. Gene transfer techniques (transfection of eukaryotic cells)

The introduction of DNA or RNA molecules into target cells has been used to create cells expressing transiently or stable a gene of interest. Transfections are important to study cellular expression of proteins, for protein localization, to study transcription or translation, and for many other purposes.

Many techniques are available to introduce DNA of eukaryotic expression vectors containing the gene of interest into mammalian cells. These techniques can be basically divided into chemical, mechanical, electrical and viral methods. The transfection methods used in my study are briefly described below.

## 2.2.20.1. Calcium phosphate precipitation method

Calcium phosphate is a well known method to introduce DNA into mammalian cells, and is used preferably for transient expression of foreign DNA, but has also been used for stable expression. This method consists of encapsulation of DNA within a calcium phosphate precipitate which then enters the cells by endocytosis (Chen and Okayama, 1988; Graham and Eb, 1973). One day prior to transfection, 3 x $10^6$ cells were plated in a 10 cm plate. To perform the experiment, we used two eppendorf tubes: in the first one, 20 μg plasmid DNA, 50 μl 2.5 M calcium chloride and water to 500 μl were added. In the second tube 500 μl BBS (pH 6.95) was added. The contents from the first tube (DNA/$Ca^{2+}$/water) was added dropwise to the second tube containing BBS, and followed by incubation at 37°C in a water bath for 15 minutes. During this incubation time, when using HEK 293 cells, the medium was replaced with 12 ml of DMEM high glucose medium containing antibiotics and FCS. After incubation, the transfection mix was added dropwise onto the cells and rocked gently to ensure even distribution of the precipitate. The cells were incubated at 37°C overnight with 3% $CO_2$. The next day cell medium was gently replaced with 12 ml of HEK 293 cell medium, and $CO_2$ was set to 5% again.

| **2 X BBS buffer** | | |
|---|---|---|
| BES/HCl | 50 | mM |
| NaCl | 280 | mM |
| $Na_2HPO_4$ | 1.5 | mM |
| (pH 6.95) | | |
| Storage at -20°C | | |

## 2.2.20.2. Transfection with GeneJuice

GeneJuice™ transfection reagent (Novagen) is a proprietary formulation of polyamines. One day prior to transfection the cells were trypsinized and 6 x $10^5$ cells were plated in each well of a 6 well plate (the cell number and medium volume were adjusted according to the cell line and plate used, for example, please see Table 2.7). The following protocol was used for transfection in a 6 well plate.

In a sterile eppendorf tube 100 μl serum-free medium plus 4 μl GeneJuice was mixed by vortexing and incubated for 5 minutes at room temperature. 2 μg DNA was added to the GeneJuice/serum-free medium mixture, gently mixed and incubated at room temperature for 15 minutes. The entire volume of DNA/GeneJuice was added dropwise to the cells in complete growth medium, the dish was gently rocked to guarantee even distribution, and the cells were incubated at 37°C, 5% $CO_2$ for 24-48 hours.

**Table 2.7. Plate size and recommended cell number for adherent cultures***

| Plate size | Number of cells plated the day before transfection (x $10^5$) | Volume of growth medium (ml) |
|---|---|---|
| 24 well | 1 | 0.5 |
| 12 well | 2 | 1.0 |
| 6 well/35 mm dish | 6 | 3.0 |
| 60 mm dish | 10 | 5.0 |
| 100 mm dish | 30 | 12.0 |

* The cell number shown here was optimized in our laboratory for HEK 293 cells and differs from the GeneJuice™ Transfection Reagent protocol.

## 2.2.20.3. Transfection with FUGENE

Fugene transfection reagent (Roche), which is a proprietary of lipids and other components supplied in 80% ethanol, was also used to transfect eukaryotic cells. One day before transfection, 3 x $10^5$ cells were plated per well in a 6 well plate. For transfection 97 μl DMEM was added into a sterile eppendorf tube and 3 μl FUGENE was directly pipetted into the medium (this

order is critical when using FUGENE reagent) and incubated at room temperature for 5 min. To the prediluted FUGENE reagent 1 μg DNA was added, mixed and incubated for 30 min at room temperature. The mixture FUGENE/medium/DNA was added dropwise onto the cells and the plates were rocked carefully to ensure even distribution and incubated according to standard procedures.

### 2.2.20.4. RNA Transfection (Qiagen)

To transfect RNAs transcribed in vitro (section 2.2.22) into mammalian cells, TransMessenger™ Transfection Reagent (Qiagen) was used. The advantage in transfecting RNA is that it is delivered into the cell's cytoplasm where it can be directly translated. By DNA transfection, the DNA transfected first has to enter the nucleus, be transcribed and then exported to the cytoplasm where it will be translated.

The following protocol is based on the TransMessenger Transfection Reagent Handbook 10/2002. Twenty four hours before transfection HEK 293 cells were plated at a density of 6 x $10^5$ cells per well in a 6 well plate, containing 2 ml of complete medium. On the day of transfection, 4 μl Enhancer R was diluted in Buffer EC-R and mixed with 2 μg RNA (the final volume was 100 μl). The mixture Enhancer/buffer/RNA was incubated at room temperature for 5 minutes. Soon after that, 8 μl of TransMessenger Transfection Reagent was added, and mixed by pipetting up and down five times. To allow complex formation, the sample was incubated for 10 minutes at room temperature. Almost at the end of the incubation time, the complete cell growth medium was removed and the cells were gently washed with pre-warmed $PBS^{++}$. 900 μl serum-free medium (without antibiotics) was added to the transfection complexes and mixed by pipetting up and down twice. The transfection complexes were added onto the cells dropwise, and the plate was rocked to ensure even distribution. Cells were cultivated at 37°C, 5% $CO_2$ for 3 hours, then the complex was removed from the cells, and washed once

gently with $PBS^{++}$. Carefully 2 ml of complete growth medium was added and the cells were cultivated under standard conditions.

## 2.2.21. Site-Directed Mutagenesis

To make mutations in the *myeov* upstream AUGs (uAUGs), the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene) was used. *Myeov* 5`UTR cloned into pGEM-T easy vector (Promega) was used as template. The method consists of PCR cycling (Table 2.8) using a mutagenic primer which anneals to denatured template DNA, and *Pfu* DNA polymerase to extend the mutagenic primer. This generates ds-DNA molecules, a template strand and a newly synthesized strand containing multiple mutations and nicks, which is sealed by components in the enzyme blend. This PCR reaction creates copies of the template DNA containing one or more mutations.

**Table 2.8 Cycling parameters**

| Segment | Cycle(s) | Temperature | Time |
|---|---|---|---|
| 1 | 1 | 95ºC | 1 minute |
| 2 | 30 | 95ºC | 1 minute |
| | | 55ºC | 1 minute |
| | | 65ºC | 7 minutes* |

* 2 minutes/Kb of plasmid length

After PCR, the reaction was digested with 1 µl of the restriction endonuclease Dpn I. This restriction enzyme targets the sequence 5`$Gm^6$ATC-3` which is specific for methylated and hemimethylated DNA (Nelson and McClelland, 1992) and therefore digests the parental template DNA, since almost all DNA isolated from *E. coli* strains is dam methylated, the newly synthesized and mutated DNA strand will not be digested.

In the last step, the digested DNA was transformed into XL10-Gold® ultracompetent cells, where the mutated closed circle ss-DNA was converted

into a duplex form *in vivo*. The mutant plasmid was transformed by a chemical method as described in section 2.2.8.2.

| **Mutagenesis reaction** | | |
|---|---|---|
| 10 x reaction buffer | 2.5 | µl |
| Water to a final volume of | 25 | µl |
| ds-DNA template | 50 | ng |
| Mutagenic primers each | 50 | ng |
| dNTP mix | 1 | µl |
| QuikChange® enzyme blend | 1 | µl |

## 2.2.22. RNA synthesis in vitro

To produce in vitro capped RNA, we used the kit mMESSAGE mMACHINE™ from Ambion. The transcription using this system uses SP6, T3 or T7 RNA polymerase. We have transcribed the plasmids pBSK, pBSK+UTR and pBSK+UTRmutated, which are under control of the T3 promoter. Prior to transcription, DNA was prepared as described below.

### 2.2.22.1. DNA plasmid preparation prior to *in vitro* transcription

The plasmids were first linearized using the restriction endonuclease Xho I. This restriction enzyme was used because the Xho I site was situated downstream of the sequence that we wanted to transcribe (*myeov* 5`-UTR and Firefly luciferase reporter gene). The restriction digestion was terminated by adding 1/20$^{th}$ volume of 0.5 M EDTA. DNA was then precipitated, washed with 70% ethanol and the DNA pellet was dried at 65°C and resuspended in sterile water. As the template could be contaminated with RNase A from the mini-preparation (section 2.2.9.1), the linearized DNA was treated with 150 µg RNase-free proteinase K plus 0.5% SDS for 30 minutes at 50°C. The plasmid DNA was then extracted with phenol/chloroform/isoamylalcohol, ethanol precipitated (section 2.2.16 and 2.2.17, respectively) and resuspended in DEPC-treated water in a concentration of 1 µg/µl.

### 2.2.22.2. *In vitro* RNA transcription reaction

The transcription reaction was performed using RNase free filter tips and sterile eppendorf tubes. The reaction described below was incubated in a thermal block at 37°C for 2 hours. The transcribed RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen). The quantity and purity was controlled using a spectrophotometer at $A_{260,}$ and the quality and size of the RNA transcribed was checked by formaldehyde agarose-gel electrophoresis.

| **RNA transcription** | | |
|---|---|---|
| Linear template DNA | 1 | µg |
| 10 x reaction buffer | 2 | µl |
| 2 x NTP/cap | 10 | µl |
| Enzyme mix | 2 | µl |
| Nuclease-free water to | 20 | µl |

### 2.2.23. Luciferase assay

To measure the reporter genes Firefly luciferase (*Photinus pyralis*) and Renilla luciferase (*Renilla reniformis*) (de Wet et al., 1987; de Wet et al., 1985) in transfected cells or made *in vitro*, the Dual-Luciferase® Reporter (DLR™) Assay System (Promega) was used. To perform the assay in transfected cells, it was necessary first to obtain the cell lysate as described below.

### 2.2.23.1. Procedure prior to the luciferase assay

The medium of transfected cultured cells was removed, and the cells were briefly washed with pre-warmed $PBS^{++}$. An appropriate amount of passive lysis buffer (table 2.9) was added onto the cells and incubated for 15 min at room temperature with gently shaking. From this cell lysate only 20 µl was used for luciferase measurement and the rest was stored at -80°C.

**Table 2.9. Volume of passive lysis buffer**

| Well Plate | 1X PLB (µl) |
|---|---|
| 6 | 500 |
| 12 | 250 |
| 24 | 100 |
| 48 | 65 |
| 96 | 20 |

## 2.2.23.2. Luciferase measurement

To assay Firefly and Renilla luciferase, we used an automated luninometer (Rosys Anthos Lucy 2) with dual dispenser, one to inject the substrate to measure Firefly luciferase, and one to inject the substrate to measure Renilla luciferase. For this purpose, 20 µl of the cell lysate was placed in a special 96 well plate. The luminometer was connected to a computer, and the lucy software was used to process the assay. The parameters were set (table 2.10) and the luciferases were measured sequentially from a single sample in the same tube. At first, Firefly luciferase was measured the by adding 100 µl of the Luciferase Assay Reagent II (LAR II), which generates a "glow-type" luminescent signal. After quantifying the firefly luminescence, the reaction was quenched, and Renilla luciferase reaction was measured by adding 100 µl of Stop & Glo® reagent, which also produces a "glow type" signal. The lucysoft program is based on the Microsoft® Excell software and therefore the measurements are all collected automatically in an excel table.

**Table 2.10. Parameters used to measure Firefly and Renilla luciferase**

| Parameters | Time (seconds) |
|---|---|
| Waiting time | 1 |
| Lag time | 2 |
| Integration time 1 | 10 |
| Lag time | 2 |
| Integration time 2 | 10 |

## 2.2.24. RNA preparation

Total RNA was isolated from cultured cells using the High Pure RNA Isolation Kit from Roche. Total RNA was either used directly for Northern Blot analysis or used as starting material to make mRNA. mRNA was prepared from total RNA using the mRNA Isolation Kit (Roche). Alternatively, mRNA was prepared directly from cultured cells using the GenElute direct mRNA miniprep kit (Sigma). All procedures for RNA extraction were performed as suggested by the manufacturer. The cultured cells were washed with prewarmed PBS, typsinized and the trypsination was stopped by adding full growth medium. Cells were spun down for 5 min at 300 x *g* and washed twice with PBS, the cells were then used for total RNA or Poly(A) RNA isolation. After RNA isolation, the quantity and purity was controlled using a spectrophotometer (section 2.2.10) and the quality was checked by agarose-gel electrophoresis and visualization on an UV-iluminator (section 2.2.14).

### 2.2.24.1. Isolation of total RNA

Cells resuspended in 200 µl PBS were mixed with 400 µl lysis/binding buffer. The lysate was pipetted into a filter tube combined with a collection tube and centrifuged for 15 s at 8000 x *g* and the flow through was discarded. DNase (90 µl DNase incubation buffer plus 10 µl DNase I) was added to the filter tube and incubated for 15 min at room temperature. To wash the RNA, 500 µl wash buffer I was added into the filter, centrifuged for 15 s and the flow through was discarded. The wash procedure was repeated twice with 500 µl and 200 µl wash buffer II, respectively. In the second wash the centrifugation time was increased to 2 min to remove all residual washing buffer. The filter tube was transferred to a sterile eppendorf tube and 50-100 µl DEPC-treated water was added and centrifuged for 1 min at 8000 x *g*. The RNA was measured in a spectrophotometer, and 1 µg was loaded onto an agarose gel (control gel) and the rest was dried using a speed vac centrifuge and the

pellet was resuspended in RNA sample buffer at a concentration of 0.5 µg/µl and stored at -80°C.

## 2.2.24.2. Isolation of mRNA

To isolate polyadenylated mRNA, the GenElute™ Direct mRNA Miniprep Kit (Sigma) was used. All steps were carried out at room temperature and centrifugation steps were at maximum speed as recommended by the manufacturer. The cell pellet (up to $10^7$ cells) was resuspended by vortexing in 0.5 ml Lysis Solution (containing 1 mg/ml Proteinase K in 40% glycerol). The cell lysate was transferred into a GenElute filtration column and spun down for 2 min and incubated for 10 min at 65°C. While the solution was still warm, 32 µl of 5 M NaCl plus 25 µl of the oligo(dT) beads were added to the digested cell lysate, and mixed thoroughly by vortexing. The lysate was incubated for 10 min to allow binding of the oligo(dT) beads to the mRNA. The complex was centrifuged for 5 min and the pellet was resuspended in 350 µl wash solution and transferred into a GenElute spin filter-collection tube, spun down for 2 min and washed twice with 350 µl Low Salt Wash Solution. The spin filter was transferred into a fresh collection tube and 50 µl Elution Solution pre-heated to 65°C was added into the spin filter, incubated for 5 min at 65°C and spun down for 2 min. The same elution step was repeated once. The mRNA was precipitated by adding 15 µl 2 M NaAc, 250 µl of ice-cold 100% ethanol and 1 µl glycogen (20 µg) and incubated overnight at -20°C. The next day the solution was centrifuged at full speed in a refrigerated centrifuge at 4°C for 30 minutes, the pellet was washed with 200 µl 70% ethanol and the pellet was dried at 65°C and resuspended in 20 µl RNA sample buffer.

| **RNA sample buffer** | | |
|---|---|---|
| Running buffer | 1 | x |
| Deionised formamide | 50 | % |
| Water | 20 | % |
| Formaldehyde (sterile filtration) | 20 | % |

## 2.2.25. Preparation of formaldehyde gel

Agarose (1.0 to 1.5 g) was mixed with 10 ml 10 x running buffer (1 x final concentration) and DEPC treated water to 80 ml. The agarose solution was incubated in a microwave until it was completely dissolved (during boiling the water volume decreases by evaporation, therefore DEPC-water was added to readjust the original volume of 80 ml). In a fume hood, the agarose was cooled down and 20 ml of 37% formaldehyde was added (formaldehyde vapor is toxic). The gel solution was poured immediately into a clean gel tray (the gel tray and comb were incubated overnight with 10% $H_2O_2$, and rinsed with DEPC treated water prior to use) and allowed to solidify for at least 30 minutes.

| **10 x running buffer** | | |
|---|---|---|
| HEPES | 500 | mM |
| EDTA . 2 $H_20$ | 10 | mM |
| Sodium acetate trihydrate | 50 | mM |
| (pH adjusted with NaOH to 7.0) | | |
| (autoclaved) | | |

## 2.2.25.1. Sample preparation for electrophoresis

Before loading samples on a formaldehyde gel, 20 µl (10 µg) of RNA in sample buffer was incubated for 10 min at 68°C and chilled on ice, briefly centrifuged and 2 µl ethidium bromide (0.5 µg/ml) was added and mixed by pipetting up and down, followed by addition of 5.5 µl 5 x loading buffer.

| **5 x loading buffer** | | |
|---|---|---|
| Bromophenolblue & xylene cyanol FF | 0.4 | % |
| Glycerol | 50 | % |
| $NaH_2PO_4$ | 0.01 | M |

## 2.2.25.2. RNA electrophoresis

The RNA samples were loaded onto a formaldehyde gel and run for about 3-4 hours at 80 V until the bromophenol blue marker almost reached

the end of the gel. The gel was observed under UV light and photographed. The gel was washed twice for 20 min with DEPC treated 20 x SSC buffer. In the second wash, the nitrocellulose membrane was briefly wetted in DEPC-$H_2O$ and incubated together with the gel in 20 x SSC buffer.

| **20 x SSC** | | |
|---|---|---|
| NaCl | 3 | M |
| Tri-Sodium citrate dihydrate | 0.3 | M |
| DEPC | 0.1 | % |
| pH 7.0 | | |

## 2.2.25.3. RNA blotting

To avoid contamination of the blotting apparatus, one whatman paper (larger than the gel) wetted in 20 x SSC was placed on the apparatus before making the transfer "sandwich". The "sandwich" was made by placing the gel on top of the large whatman paper. Used x-ray films were placed alongside the gel to guarantee that 20 X SSC pass through the gel only. The nylon membrane was placed onto the gel and all possible bubbles were removed. Carefully two whatman papers wetted in 20 x SSC, two dry whatman papers and tissue papers (the contents of a full box) were placed on top of the membrane and the sandwich was weighted with a one liter bottle of water.

The blot was performed until all tissues were wetted with buffer, usually an overnight incubation was enough. The next day, transfer quality was checked by UV illumination and the slots were marked on the membrane. The membrane, with the RNA facing upwards was UV-crosslinked for 12 seconds (auto crosslink setting, 254 nm, Stratagene, Stratalinker). The membrane was then placed inside a whatman paper and baked for 2 hours at 80°C.

## 2.2.25.4. Hybridization

The DNA insert to be labeled (50 to 100 ng) was diluted in a total volume of 22 μl water and incubated at 95°C for 5 min and chilled on ice for 2

min. To avoid DNA precipitation, the following solutions were warmed at 37°C for 2 min, and added to the DNA (which was also warmed at 37°C).

| **HexaLabel™ DNA Labelling Kit (MBI Fermentas)** | | |
|---|---|---|
| Hexanucleotide in 5 x buffer | 10 | µl |
| Spermidin (10 mM) | 3 | µl |
| Mix minus C | 3 | µl |
| $^{32}$P α-dCTP | 4 | µl |
| Polymerase klenow | 1 | µl |

The reaction was incubated at 37°C for 10 min, 4 µl dNTPs were added and incubation was proceeded at 37°C for 5 min. The reaction was stopped by adding 1 µl 0.5 M EDTA.

The reaction was applied to a Sephadex G50 column and then 80 µl of TE buffer was added dropwise, followed by the addition of 130 µl of TE buffer twice. The flow through was collected in one eppendorf tube. This was followed by a successive application of 130 µl TE buffer (not dropwise any more) of which each flow through was collected in a single eppendorf tube. The radioactivity was measured and the reaction containing the radiolabelled DNA probe was taken for further experiments. To this solution, 20 µl salmon sperm DNA was added, followed by incubation for 10 min at 95°C and cooled on ice. The solution was finally added to the hybridization mix, swirled 10 times before the membrane was added and incubated overnight at 63°C.

| **Hybridization mix** | | |
|---|---|---|
| Denhardt´s | 10 | x |
| Dextransulfat | 10 | % |
| SDS | 0.1 | % |
| Salmon sperm DNA | 50 | mg/l |

| **100 x Denhardt´s** | | |
|---|---|---|
| Ficoll type 400 | 2 | % |
| BSA fraction V | 2 | % |
| Polyvinylpyrolidon type 360 | 2 | % |

## 2.2.25.5. Washing and membrane exposure

On the following day, the membrane was washed with 3 x wash buffer for 20 min at 63°C. When necessary the membrane was washed more

stringently (1 x, 0.3 x or 0.1 x wash buffer) and/or the temperature was increased up to 73°C. The membrane was placed in a MS-cassete with intensifying screen containing MS-film and kept at -80°C and developed the following day.

## 2.2.26. Electrophoresis of proteins on SDS-polyacrylamide gels

To separate proteins by size, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a vertical gel chamber according to a described method (Towbin et al., 1979). This method is based on a two gel layer system (Laemmli, 1970): a stacking gel and a separating gel.

| **Solution for preparing 12% separating gels** | | |
|---|---|---|
| Water | 5.15 | ml |
| 40% Acrylamide:bisacrylamide 29:1 | 3.60 | ml |
| 1.5 M Tris/HCl, pH 8.8 | 3.00 | ml |
| 20% SDS | 60 | µl |

| **Solution for preparing 5% stacking gels** | | |
|---|---|---|
| Water | 4.66 | ml |
| 40% Acrylamide:bisacrylamide 29:1 | 780 | µl |
| 1.0 M Tris/HCl, pH 6.8 | 800 | µl |
| 20% SDS | 32 | µl |

### 2.2.26.1. Gel preparation

The amounts depicted above are sufficient to make 2 mini gels in a cassette from Invitrogen. The contents were mixed and in order to polymerize the separating gel, 10% ammonium persulphate (120 µl) plus TEMED (7.2 µl) was added. Working quickly, the solution was applied into the gel cassette up to ± 6.5 cm, and the last ± 2.5 cm of the cassette was filled with distilled water. The gel was polymerized for at least 30 min, and the water was carefully removed. To prepare the stacking gel, 64 µl APS and 6.4 µl TEMED were added to the stacking gel solution and the solution was directly poured into the gel cassette, and the gel comb was placed to form the slots. The stacking

gel was polymerized for at least 30 min, and used on the same day or stored at 4°C in a sealed bag containing 1 x running buffer.

### 2.2.26.2. Sample preparation

The samples containing proteins were resuspended in 1 x sample buffer (with 10% DTT). DTT is prepared in 0.01 M Na-acetate pH 5.2 and added at a final concentration of 10% to the sample just prior to use. The samples were denaturated for 5 min at 95°C and loaded immediately on the SDS PAGE gel.

| **4 x Sample buffer** | | |
|---|---|---|
| Tris/HCl pH 6.8 | 0.2 | M |
| SDS | 8 | % |
| Bromophenol | 0.02 | % |
| Glycerol | 40 | % |
| DTT | 0.1 | M |

### 2.2.26.3. Electrophoreris

Electrophoresis was carried out in Tris-glycine electrophoresis buffer using a Novex apparatus. The electrophoresis was divided in two steps. During the first step the proteins run in the stacking gel for 15 min at 150 V, 250 mA, 25 W. In the second step, the proteins were separated by size running through the pores of the separating gel for 105 min at 100 V, 250 mA, 25 W. As molecular weight marker SeeBlue®Plus2 from Invitrogen was used, containing marker proteins of 4, 6, 16, 30, 36, 50, 64, 98 and 250 KDa, respectively. The protein gel was ready for blotting and subsequent immunodetection (sections 2.2.26.4 and 2.2.26.5, respectively).

### 2.2.26.4. Transfer of proteins from gels to nitrocellulose membrane

For immunological detection of the proteins of interest, the proteins from the gels were transferred after electrophoretic separation onto a nitrocellulose membrane. For this purpose, a transfer set (depicted below)

was prepared using: 2 sponges, the gel from SDS-PAGE, nitrocellulose membrane, and 4 whatman papers (cut according to the gel size).


All contents were soaked in transfer buffer. To prepare the transfer set, the first sponge and two whatman papers were placed inside the transfer plate (cathode side), and the gel was carefully placed on top of the whatman paper. The nitrocellulose membrane was then placed on the gel, which was covered with the last two whatman papers and the sponges, and finally closed with the transfer plate (anode side). The transfer set was placed inside the transfer apparatus immersed with transfer buffer, and the electric field was applied (25 V, 125 mA, 17 W, for 90 min). After transfer, the gel was stained to check for blotting efficiency and the nitrocellulose membrane was used for immunodetection (section 2.2.26.5).

| **Transfer buffer** | | |
|---|---|---|
| Tris Base | 12 | mM |
| Glycine | 96 | mM |
| Methanol | 20 | % |
| (pH 8.3 prior addition of methanol) | | |

## 2.2.26.5. Immunodetection of blotted proteins

Immunodetection of specific proteins blotted onto nitrocellulose membrane was performed according to Gershoni, JM (Gershoni and Palade, 1983). Nitrocellulose membrane with immobilized proteins were first stained with Ponceau Red (0.1% v/v Ponceau S in 1% v/v acetic acid) to control the amount of proteins present on the membrane, and to mark the molecular weight markers. The membrane was washed with distilled water, and it was

subsequently blocked for 1 hour at RT in blocking solution (10% dry milk in TBS-T) to block free binding sites on the membrane. The membrane was then incubated overnight at 4°C with the appropriate antibody (diluted in blocking solution) which recognizes and binds to the protein of interest. Next day, unbound antibodies were washed off (5 x 6 min) with 1 x TBS-T buffer, and the membrane was then incubated with the secondary antibody (goat IgG against rabbit IgG or mouse IgG conjugated with horseradish peroxidase) for 1 hour at room temperature. After washing off unbound secondary antibody conjugates (5 x 6 min) with 1 x TBS-T buffer, positive bands were detected using the chemiluminescence reaction (section 2.2.26.6).

| **Antibody** | **Type** | **Company** | **Dilution** |
|---|---|---|---|
| Anti-GAL4 BD | mouse monoclonal | Santa Cruz | 1 : 10,000 |
| Anti-MYEOV | rabbit polyclonal | Homemade | 1 : 1,000 |
| Goat anti-mouse | IgG-HRP conjugated | BioRad | 1 : 5,000 |
| Goat anti- rabbit | IgG-HRP conjugated | BioRad | 1 : 20,000 |

## 2.2.26.6. Cheminulescence reaction

To visualize the proteins, an ECL kit was used as recommend by the manufacturer (Amersham). An equal volume of detection solution 1 (oxidizing reagent) was mixed with detection solution 2 (enhancer reagent), and used to cover the nitrocellulose membrane for 1 min. Subsequently, the membrane was placed in the film cassette. The lights were switched off and a sheet of autoradiography film (X-OMAT AR, Kodak) was placed on top of the membrane. The cassette was closed and the membrane was exposed to a film for 1 min. The film was developed immediately in a film developer (Epichemi II darkroom, UVP). On the basis of the appearance of various bands it was estimated how long the exposure of the second film had to be continued. Second exposures varied from 20 sec to 5 hours, depending on the amount of the target protein on the membrane.

# 3. Results

## 3.1. Translation of *myeov* open reading frame

The *myeov* gene codes for a transcript of 2300 nucleotides and exhibits a long 5`UTR of 445 nucleotides and a 3`UTR of 800 nucleotides. The *myeov* mRNA contains two open reading frames coding for two proteins, one long one with 313 amino acids and one short one with 255 amino acids. Both proteins share the same stop codon (Figure 3.1).

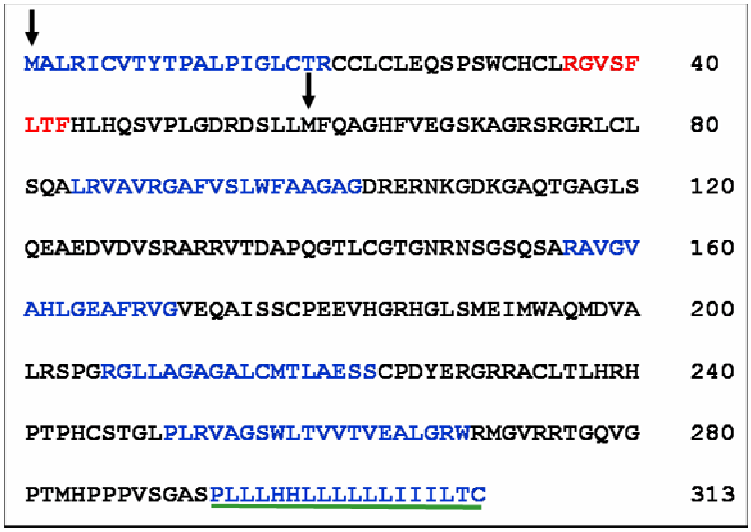



**Figure 3.1. The amino acid sequence of *myeov*.**
The possible two open reading frames of *myeov* are shown, the arrows indicate the start codon of both proteins, that share the same stop codon. The six transmembrane domains are shown in blue, and the ribonucleoprotein-1 motive is marked red. The leucine and isoleucine tail is underlined.

The long open reading frame has a start codon in a suboptimal context (CTC**AUG**G) and the second open reading frame has an imperfect start codon (CTC**AUG**T) (for details of Kozak's start codon, please see the introduction section 1.4).

## 3.2. The complete *myeov* mRNA is not translated

Several constructs containing either the full *myeov* cDNA or only the long or short open reading frame were cloned into the eukaryotic expression vector pMT2SM (Figure 3.2a). The eukaryotic expression vector pMT2SM

containing *myeov* constructs were transfected into several eukaryotic cell lines to transiently express MYEOV. Three days after transfection of the constructs shown on Figure 3.2a, proteins were isolated from the transfected cells and were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membrane by standard procedures. The nitrocellulose membrane was blocked, and incubated with our MYEOV specific antibodies and developed using a luminescence reaction (Figure 3.2b).

The protein detected with our MYEOV antibody in lane 1 (Figure 3.2b) corresponds to transfection of DNA of construct number 1, where the *myeov* short open reading frame was modified to have a perfect Kozak's start codon (shown with a green arrowhead). The second lane shows the protein corresponding to construct number 2. In this construct the start codon of the long open reading frame was modified to a perfect Kozak's start codon. Proteins shown in lanes three and four correspond basically to the constructs one and two, however, these constructs lack the leucine tail (see Figure 3.1, underlined amino acid sequence). Lanes five and six show transfections with constructs of the *myeov* short and long ORF containing its own start codon, again protein could be detected using our MYEOV specific antibody. In the lanes 1 to 6, we can see that the transfected constructs produce enough *myeov* proteins detectable by MYEOV antibody. However, in lanes 7 and 8 no-protein could be detected. These lanes correspond to constructs exhibiting the complete *myeov* cDNAs coding for the short and the long ORF, respectively. The absence of MYEOV protein after transfection with constructs containing the full *myeov* cDNA draws our attention. We wondered why transfection of the full *myeov* cDNA did not produce detectable MYEOV proteins. During standard ribosome scanning, the 40S ribosomal subunit, with associated initiation factors bind in the vicinity of the cap structure in the 5`UTR and scans the mRNA in the 3`direction until an initiation codon in a favorable context is reached, and protein translation is initiated (Kozak, 1999). In line with this knowledge, our experiments suggested that the *myeov* 5`UTR was responsible for the lack of MYEOV protein translation.

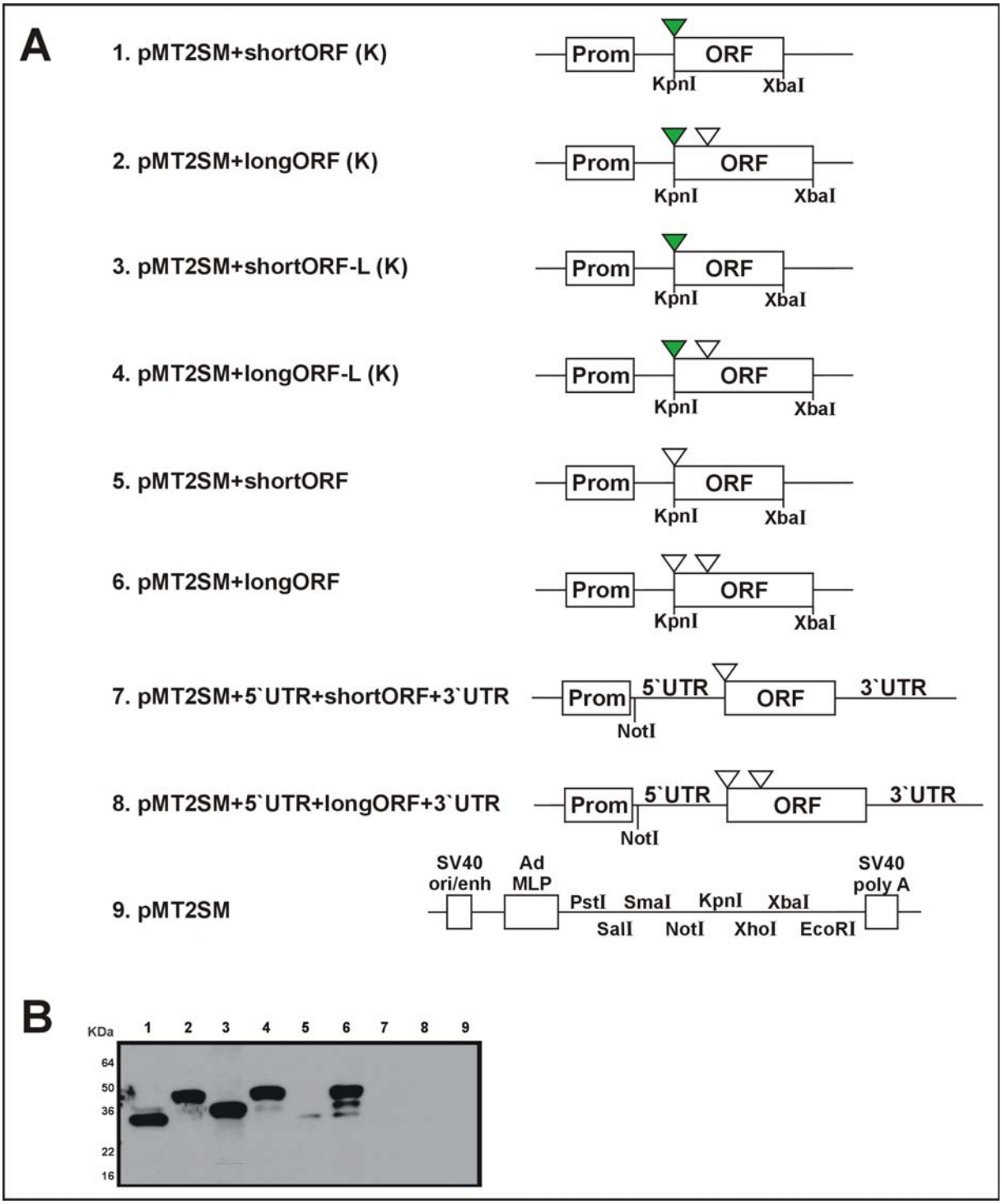



**Figure 3.2. Translation efficiency of *myeov* cDNA constructs.**
(A) Different *myeov* open reading frame (ORF) cDNA fragments (constructs 1-6) were inserted using the indicated restriction sites into the eukaryotic expression vector pMT2SM (construct 9). The green arrowhead indicates a start codon in a perfect Kozak context. An open arrow indicates the start codon of the original first or second ORF, respectively. The constructs number 7 and 8 contain the entire *myeov* cDNA. B) The constructs were transiently transfected into HEK 293 cells, and 48 hours after transfection the cells were lysed and Western immunoblotting was performed. Specific MYEOV polyclonal antibodies were used. Note that no MYEOV protein could be detected in cells transfected with DNA constructs containing the full *myeov* cDNA.

## 3.3. Structural features of the *myeov* 5`UTR

The *myeov* 5`UTR exhibits an unusual long length of 445 nucleotides, containing four AUG codons, which code for four possible open reading frames of respective 22, 59, 11 and 7 amino acids. These upstream AUGs (uAUGs) are located upstream of the translation start site of the open reading frame. Concerning the relevance of nucleotides at the position -3 and +4 in the Kozak's consensus sequence for optimal ribosome recognition of a start codon, the first three uAUGs in *myeov* 5`UTR show only one of the two Kozak's features. The fourth uAUG shows both features, however the ORF initiated from this AUG codes for a very small peptide of only 7 amino acids (Figure 3.3).

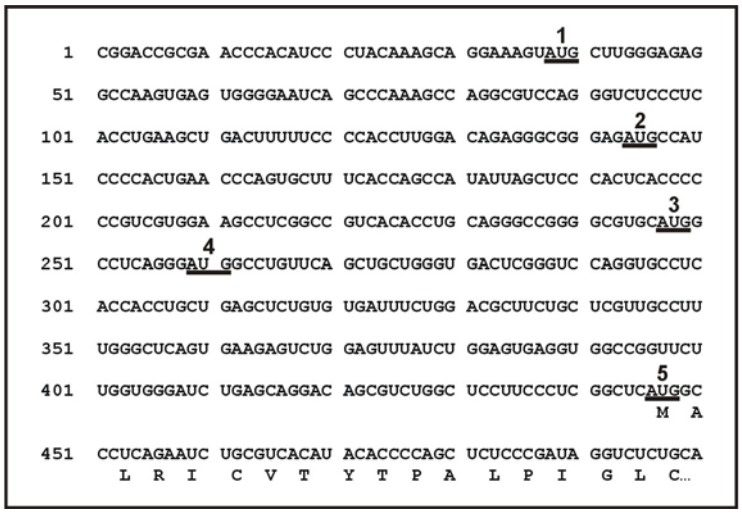



**Figure 3.3. The *myeov* 5`UTR cDNA sequence.**
The *myeov* 5`UTR contains four upstream AUG codons that are indicated in the order of its appearance from number 1 to 4. The start codon of the main *myeov* open reading frame is also indicated (AUG number 5). The first 18 amino acids of the *myeov* protein are shown in one letter code.

Analyzing the *myeov* 5`UTR sequence to structure prediction using the software mfold 3.1 algorithm (Zuker, 2003), we found that *myeov* 5`UTR was predicted to have a very complex secondary structure with several stable hairpins and a Gibbs free energy of $\Delta G$ of -153,1 kcal/mol for the most stable configuration (Figure 3.4).

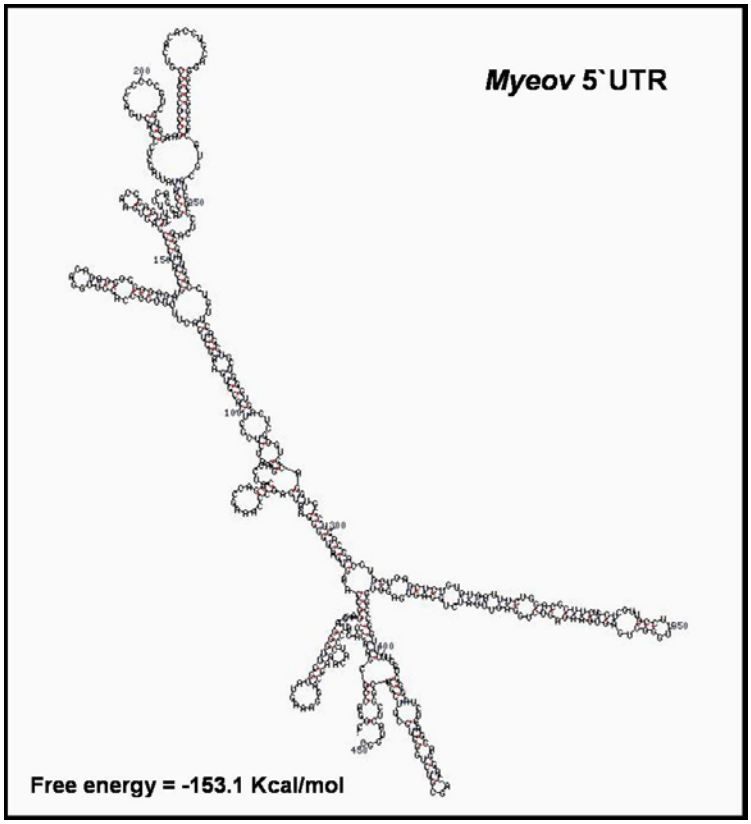



**Figure 3.4. Predicted *myeov* secondary structure.**
The mRNA of the *myeov* 5`UTR was submitted to the software mfold 3.1 and predicted to have a stable secondary structure containing several hairpins. The most stable configuration with a free energy of -153.1 kcal/mol.

It has been described that mRNAs containing a secondary structure with a folding free energy of ≤50 kcal/mole can effectively reduce ribosome scanning or even promotes complete inhibition of the scanning mechanism. Our data suggest that *myeov* mRNA translation is strictly regulated and MYEOV protein overproduction is repressed. However, it is possible that when MYEOV proteins are required, translation might occur by a cap-independent mechanism, e.g., internal ribosome entry.

## 3.4. Effect of *myeov* 5`UTR on translation of a downstream reporter gene

In order to verify the effect of the *myeov* 5`UTR on translation of a reporter gene, we amplified *myeov* 5`UTR and added the restriction sites EcoRI and NcoI using the primers myeovEcoRI and myeovNcoI. This PCR

fragment was cloned into pGEM-T easy vector, the cloned fragment was checked by sequencing and subcloned between EcoRI/NcoI sites located upstream of the reporter gene Firefly luciferase in the monocistronic plasmid pGL3 (Promega), and thus creating the vector pGL3+UTR (Figure 3.5a). The Firefly luciferase present in the vector pGL3 has a perfect start codon. However, in order to mimic the normal situation as found in *myeov* cDNA, we introduced the same suboptimal start codon as found in the *myeov* long open reading frame. For that, the perfect start codon was modified to an suboptimal start codon after cloning of *myeov* 5`UTR into the vector pGL3. Considering the possibility that modification of the Kozak's start codon might result in poor translation of the Firefly luciferase, we designed another set of primers to amplify *myeov* 5`UTR exhibiting a perfect start codon. For that the *myeov* 5`UTR was amplified using myeovEcoRI and myeovNcoI-K oligonucleotides primers, cloned into pGEM-T easy vector, checked by sequencing and subcloned into the correct open reading frame of the Firefly luciferase cistron in the plasmid pGL3, creating pGL3+UTR(K) (Figure 3.5a). These vectors were transfected into HEK 293 cells, and 48 hours after transfection, cells were lysed with lysis buffer provided with the Dual luciferase Kit (Promega). The Firefly luciferase light units were measured using an automated luminometer (Anthos) machine (Figure 3.5b). As we can see in Figure 3.5b, the presence of the *myeov* 5`UTR in both constructs (pGL3+UTR and pGL3+UTR(K) strongly represses translation of the reporter gene Firefly luciferase activity by 93% and 96%, respectively (Table 3.1). This result was in accordance with the results obtained after transfection of a complete *myeov* cDNA construct in a eukaryotic expression vector into HEK 293 cells (Figure 3.2b, lanes 7 and 8).

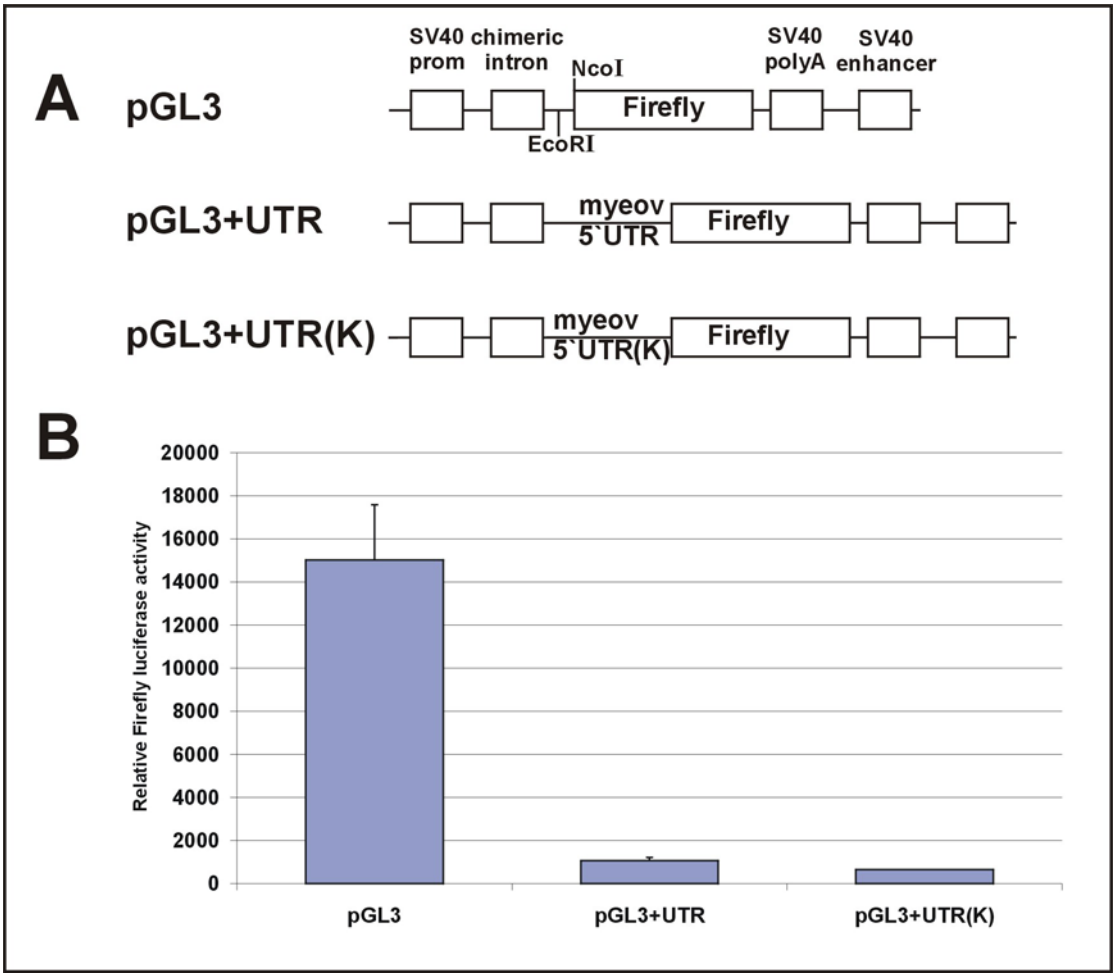



**Figure 3.5. The *myeov* 5`UTR reduces translation initiation efficiency of a downstream reporter gene.**
(A) The *myeov* 5`UTR was inserted in the indicated restriction sites into the monocistronic vector pGL3, creating the constructs pGL3+UTR and pGL3+UTR(K). In the construct pGL3+UTR the start codon of the Firefly luciferase was modified to a suboptimal context to mimic the *myeov* start codon, whereas the start codon of the Firefly luciferase in the construct pGL3+UTR(K) contains an optimal Kozak context. (B) These DNA constructs were transfected into HEK 293 cells and the relative activity of Firefly luciferase was measured.

Our results clearly show that the presence of the *myeov* 5` UTR blocks protein translation of the reporter gene, implicating that *myeov* 5`UTR may control protein translation. In analogy to that what has been described for other genes, it may pass that *myeov* might be translated under specific situations (e.g. heat shock, apoptosis, etc). In this situation, the ribosomes would escape the strong secondary structure found in *myeov* 5`UTR mRNA (Figure 3.4) and jump directly at or near the AUG of the main ORF. This cap-independent translation mechanism has been called Internal Ribosome Entry Site mediated translation (IRES). To test this possibility, an

inverted sequence (hairpin) with -55 kcal/mole was introduced downstream of the promoter region in the vector pGL3 to create the vector phpL. The same was performed in the vector pGL3+UTR and pGL3+UTR(K), creating the vectors phpL+UTR and phpL+UTR (K), respectively (Figure 3.6a).

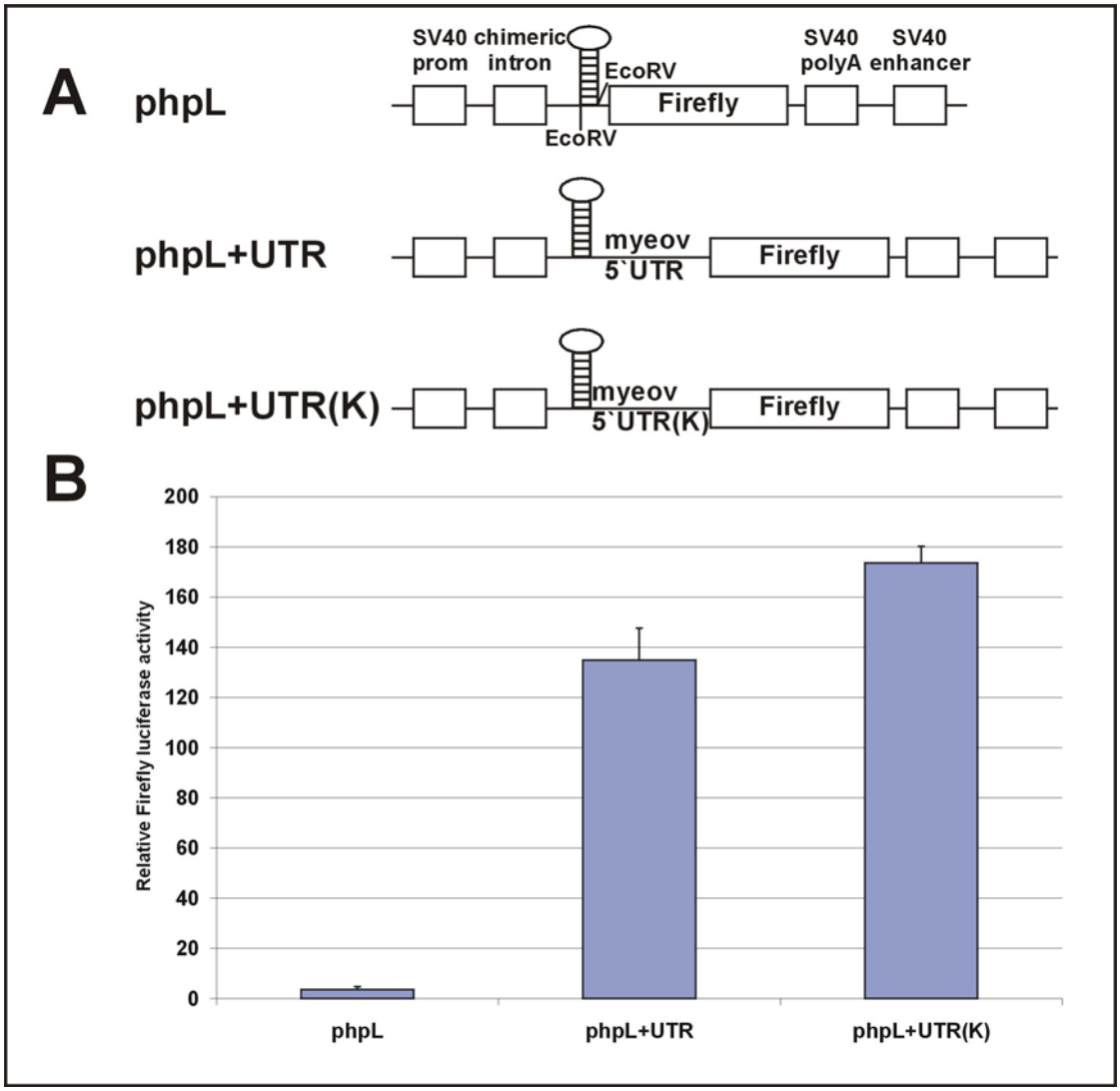



**Figure 3.6. *Myeov* 5`UTR directs internal ribosome entry in the presence of a stable hairpin.**
(A) A palindromic sequence that when transcribed creates a stable hairpin with a free energy of -55 kcal/mol and blocks ribosomal scanning was inserted at the indicated site in the DNA constructs depicted in Figure 3.5, creating phpL, phpL+UTR and phpL+UTR(K). (B) The monocistronic DNA constructs were transiently transfected into HEK 293 cells and Firefly luciferase activity was measured.

The presence of a stable hairpin blocks the ribosome scanning and therefore the translation of the Firefly luciferase reporter gene should be severely impaired. In case the *myeov* 5`UTR contains, for example an Internal Ribosome Entry Site, the ribosome can bind to the IRES, and Firefly luciferase will be translated. The DNA constructs depicted in Figure 3.6a were transiently transfected into HEK 293 cells and luciferase was measured 48 hours after transfection (Figure 3.6b). Our results show that the presence of a hairpin inhibits translation of the reporter gene by ~99% and emphasizes its effectiveness in blocking ribosome scanning. However, after transfection of phpL+UTR and phpL+UTR(K), Firefly luciferase activity was 38 and 48-fold higher as compared to the empty vector phpL. These data suggest that the Firefly luciferase produced was translated by a cap independent manner. e.g. IRES-mediated translation.

## 3.5. Does the *myeov* 5`UTR harbors an Internal Ribosome Entry Site?

To answer this question, *myeov* 5`UTR was inserted into the bicistronic vector pRF between the Renilla luciferase (Rluc) and Firefly luciferase (Fluc) reporter genes. The first cistron (Renilla) is under control of a simian virus (SV40) promoter and is translated via a cap dependent mechanism. The Renilla luciferase is used to correct for transfection variation. The second cistron (Firefly luciferase) is translated independent of the cap structure, e.g. by internal ribosome entry site. *Myeov* 5`UTR fragment was obtained from the vector pGEM-T+UTR and pGEM-T+UTR (K) and cloned upstream of the Firefly luciferase cistron originating pRF+UTR and pRF+UTR(K), respectively (Figure 3.7a). These vectors were transiently transfected into HEK 293 cells and 48 hours after transfection, activities of both luciferases were measured using the Dual Luciferase Kit from Promega in an automated luminometer with dual dispenser. Figure 3.7b demonstrates that the Firefly luciferase activity was increased 6- and 9-fold in pRF+UTR and pRF+UTR(K), respectively, when compared to the empty vector pRF.

This result suggests the possibility that the *myeov* 5`UTR may contain an IRES. However, one has to consider other possible mechanisms that may lead to the production of Firefly luciferase activity after transfection of bicistronic constructs. For example, the Firefly luciferase activity observed using the bicistronic assay may be due to ribosome shunting. In this situation, the ribosome goes to a downstream site by intramolecular shunting, bypassing the RNA structure, and resumes scanning until the next appropriate start codon is reached (Hellen and Sarnow, 2001).

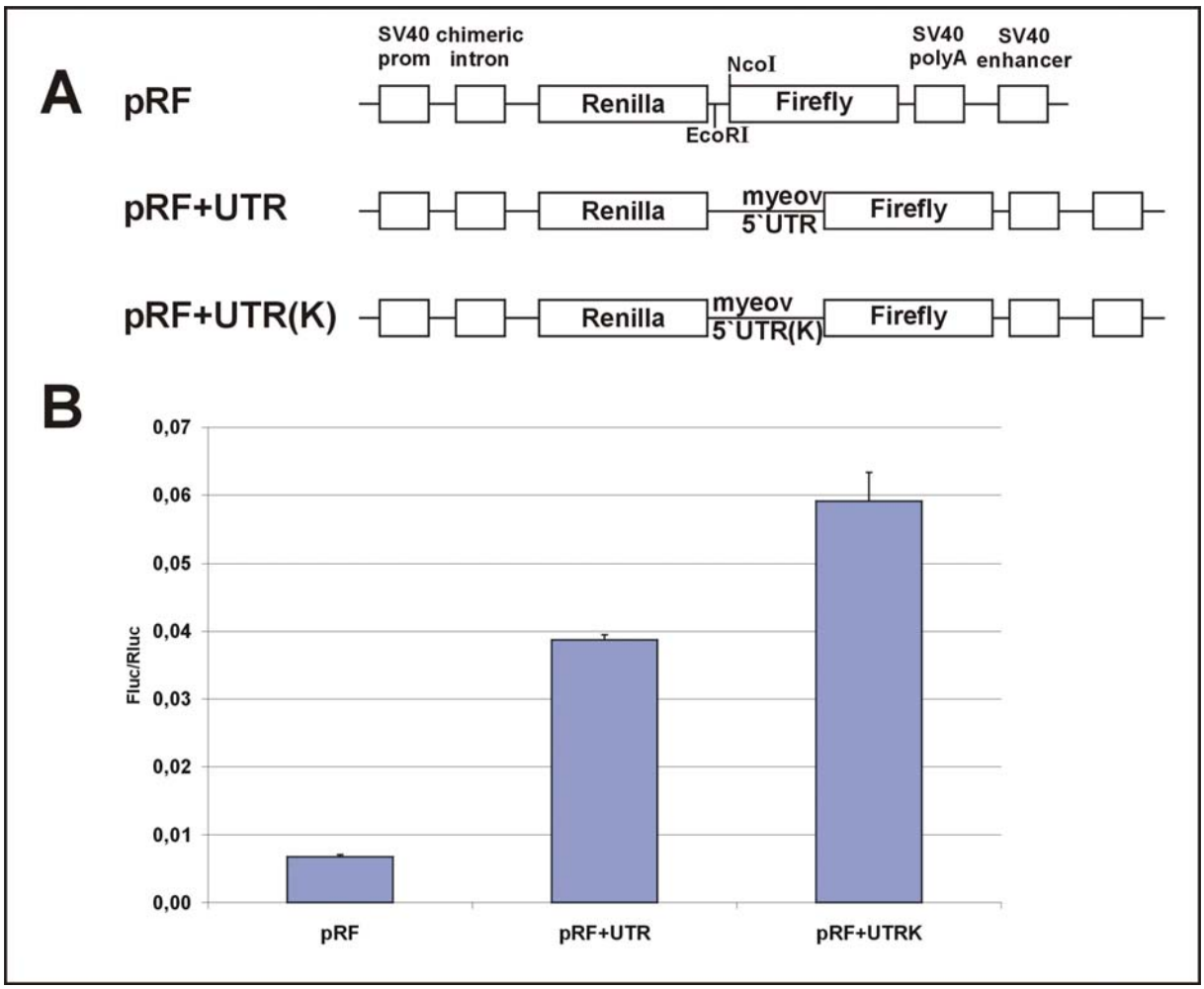



**Figure 3.7. *Myeov* 5`UTR supports translation of the 3`cistron in a bicistronic vector.** (A) The bicistronic expression cassette of the vector pRF. The *myeov* 5`UTR sequence was inserted into pRF using the indicated restriction sites to create the construct pRF+UTR and pRF+UTR(K). In the construct pRF+UTR the start codon of the Firefly luciferase was modified to a suboptimal Kozak context to mimic the *myeov* start codon. The Firefly luciferase start codon in the construct pRF+UTR(K) contains the optimal Kozak start codon. (B) HEK 293 cells were transfected with these DNA constructs and the activities of both luciferases were measured and normalized to internal control Renilla luciferase levels.

To eliminate this possibility and in order to demonstrate that the observed Firefly luciferase was indeed due to IRES activity, an inverted repeated sequence (hairpin) was placed upstream of the Renilla luciferase open reading frame of the vectors pRF, pRF+UTR and pRF+UTR(K), thus creating the constructs phpRF, phpRF+UTR and phpRF+UTR(K), respectively (Figure 3.8a).

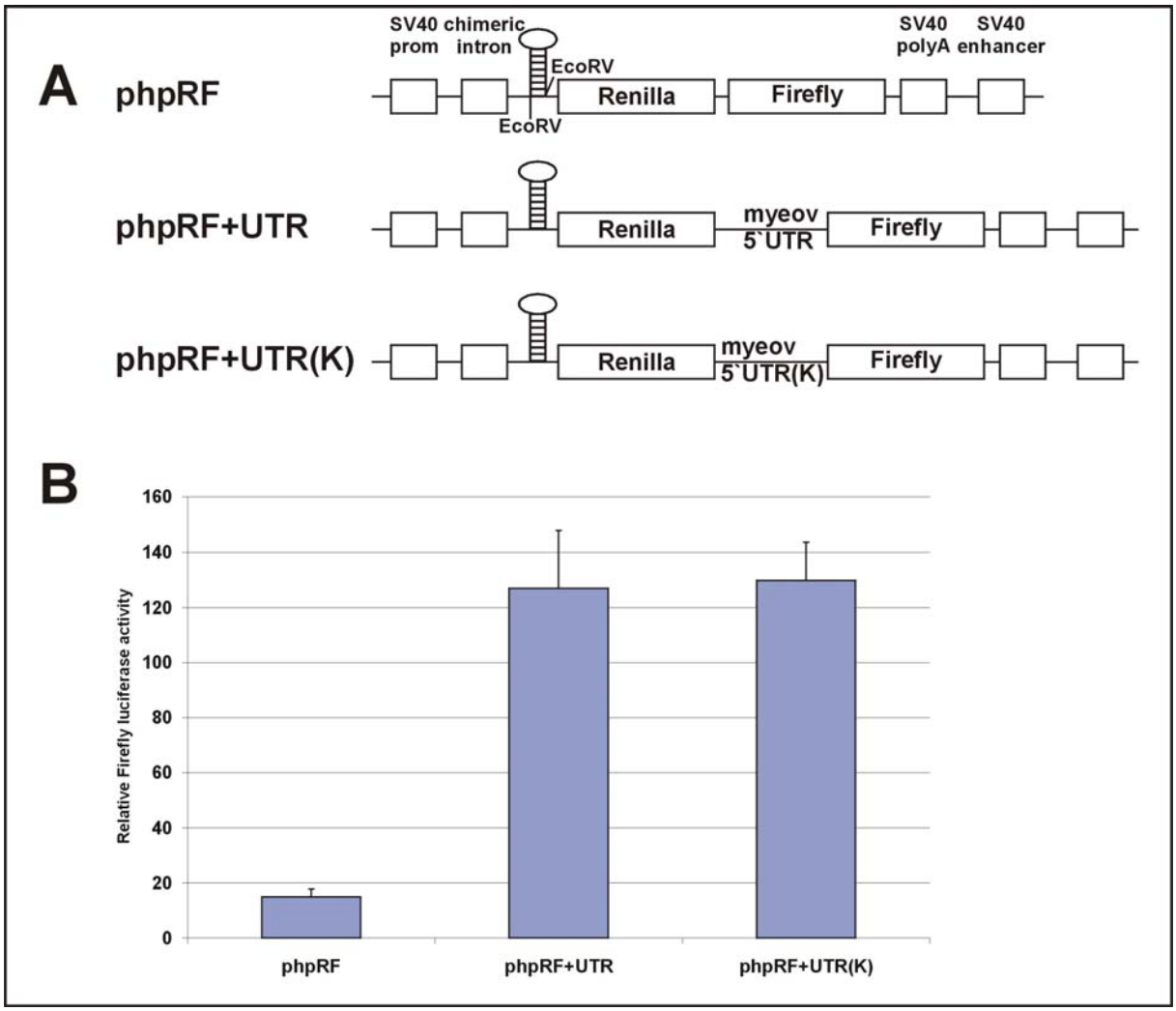



**Figure 3.8. A stable hairpin appended to 5`end of bicistronic mRNA does not block translation of the 3`cistron.**
(A) A palindromic sequence that when transcribed generates a stable hairpin with free energy of -55 kcal/mol was inserted upstream of the Renilla luciferase cistron in the constructs depicted in Figure 3.7: pRF, pRF+UTR and pRF+UTR(K), creating phpRF, phpRF+UTR and phpRF+UTR(K). (B) HEK 293 cells were transiently transfected with these DNA constructs and both reporter gene activities were measured. Firefly luciferase activities were normalized to control Renilla luciferase levels.

These constructs were transiently transfected into HEK 293 cells and 48 hours after transfection cells were lysed and luciferase activities were measured (Figure 3.8b). As expected, insertion of a hairpin effectively blocks ribosome scanning, and Renilla luciferase activity produced from these constructs was reduced to 90-100% of that of wild type (pRF). Again introduction of the *myeov* 5`UTR stimulated Firefly luciferase activity. This result suggests that translation of Firefly luciferase in the bicistronic constructs were independent of ribosome scanning from the 5`end of the bicistronic mRNA, and may probably occur by a cap independent mechanism (e.g. IRES).

## 3.6. *In vitro* Coupled Transcription and Translation

In order to confirm that *myeov* 5`UTR has an Internal Ribosome Entry Site, we performed an additional control experiment. In this experiment, transcription starts from a T7 promoter and translation is performed *in vitro* in a rabbit reticulocyte lysate. In addition to the SV40 promoter, the bicistronic vector also contains a T7 promoter located downstream of the SV40 promoter (Figure 3.9a). In this assay translation of Renilla luciferase still occurs via cap-dependent scanning, whereas translation of Firefly luciferase still depends on the presence of an IRES in the intercistronic region. Firefly luciferase activity should be expected when an IRES is present. Both constructs pRF and pRF+UTR were transcribed and translated *in vitro* using a Coupled Transcription and Translation Kit (Promega). The *in vitro* reaction was performed in a total volume of 50 µl and every 10 minutes a sample of 5 µl was taken from the reaction and mixed with 2 x Passive Lyses Buffer (Promega), and reporter activity of Renilla and Firefly luciferase was measured (Figure 3.9b). The Renilla luciferase (used as internal control) activity was high showing that the reactions worked properly. However, to our surprise the results demonstrated basically no difference between the Firefly luciferase activity of the empty bicistronic vector and the bicistronic construct comprising the *myeov* 5`UTR (Figure 3.9b).

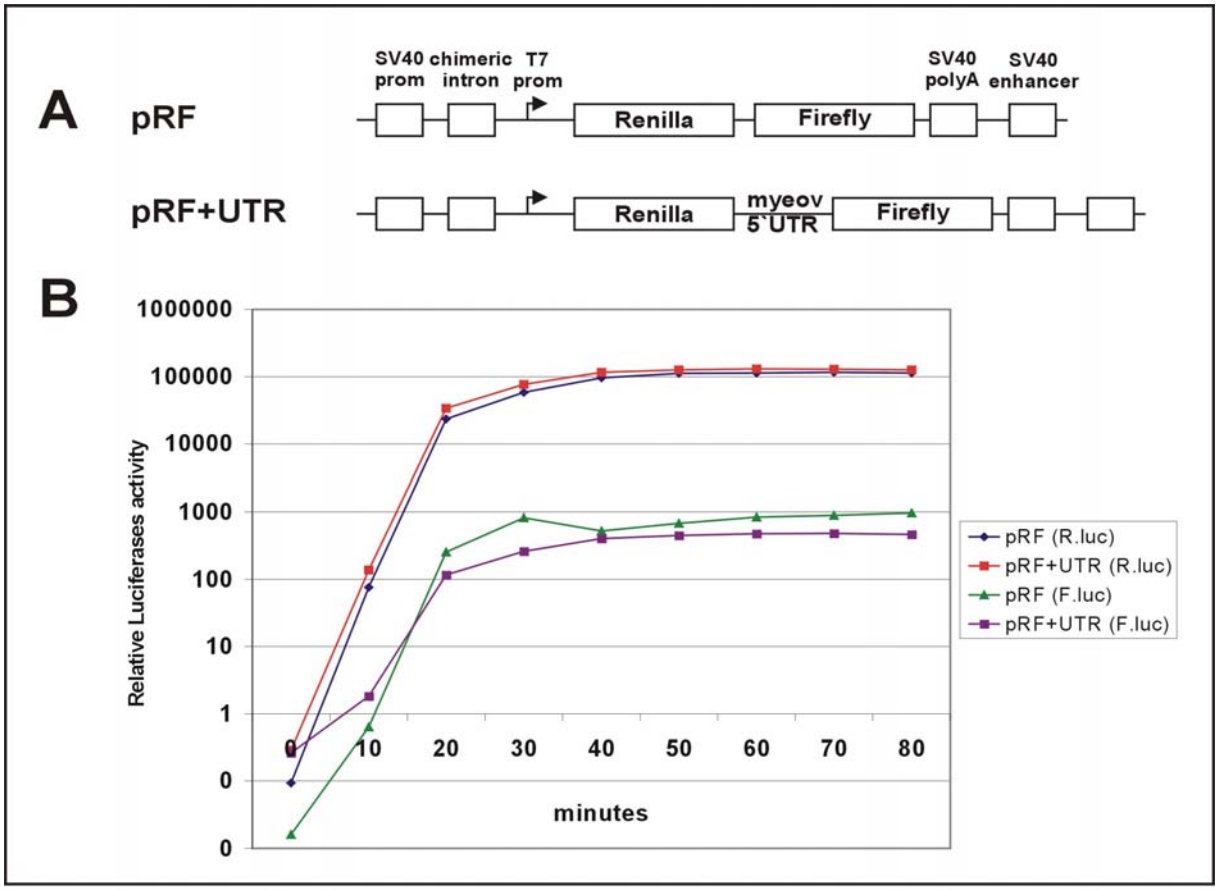



**Figure 3.9. Effect of the *myeov* 5`UTR on reporter gene expression *in vitro*.**
(A) Bicistronic constructs pRF and pRF+UTR. The arrow indicates the position of the T7 promoter. (B) These constructs were transcribed and translated *in vitro* using T7 polymerase for transcription of the RNA and a nuclease-treated rabbit reticulocyte lysates to translate the transcribed RNA. Every 10 minutes 5 µl of the sample was mixed with lysis buffer and used to measure both luciferases activities. The Renilla luciferase activity is used as internal control and the Firefly luciferase activity is used to measure the putative IRES activity. R.luc = Renilla luciferase, F.luc = Firefly luciferase.

We also used the known IRES sequence of the encephalomyocarditis virus (EMCV) cloned into the vector pRF upstream of the Firefly luciferase cistron as a positive control (pRF+EMCV, Figure 3.10). In this construct the Renilla luciferase is translated via a cap-dependent mechanism and the Firefly luciferase is translated via the IRES of EMCV. We used pRF, pRF+UTR and pRF+EMCV in a coupled *in vitro* transcription and translation assay. Every 10 minutes one tenth of the sample was mixed with 2 x Passive Lysis Buffer, and luciferases activity were measured (Figure 3.10b and c).

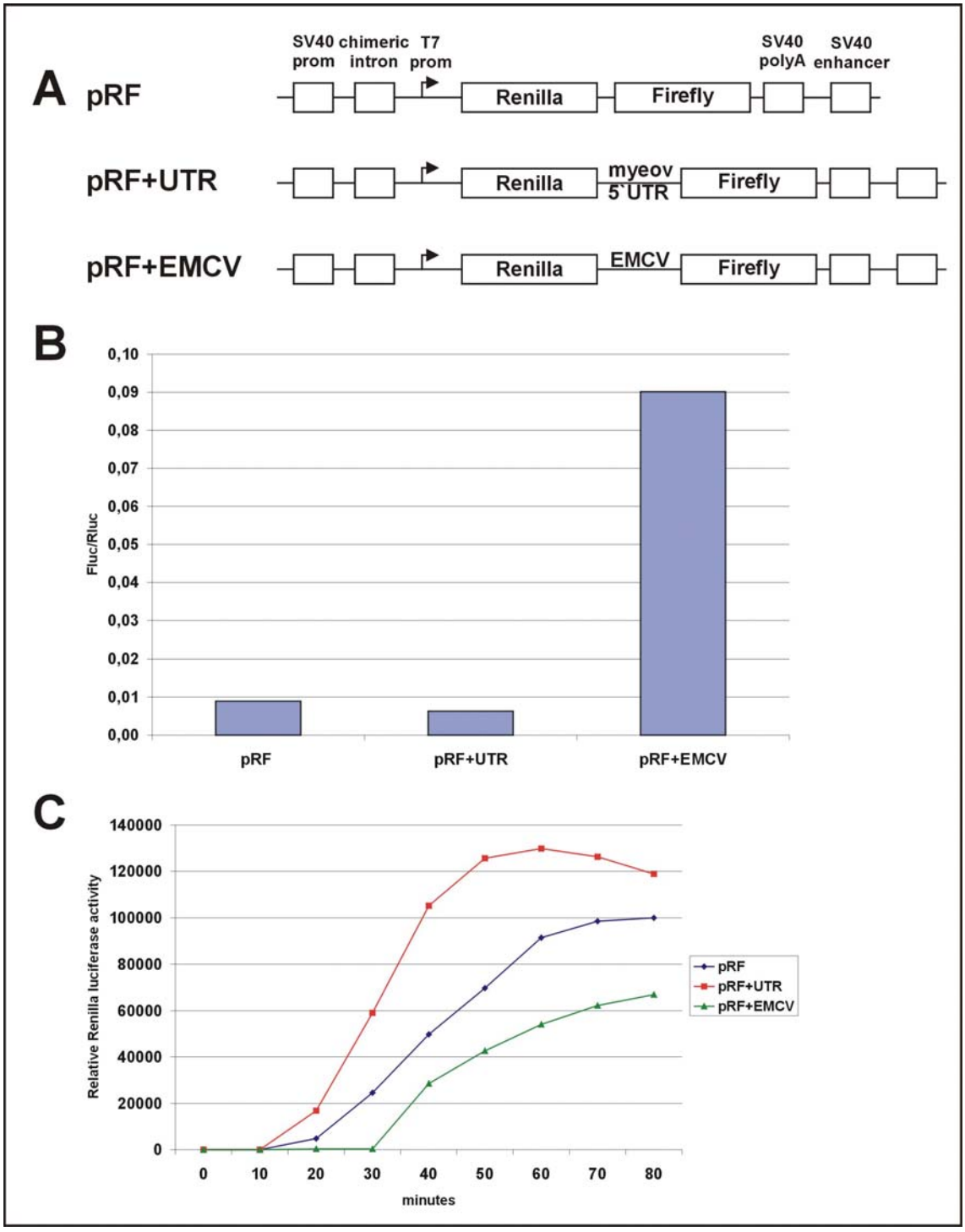



**Figure 3.10. Effect of the *myeov* and *EMCV* 5`UTRs on reporter gene expression *in vitro*.**
(A) Schematic diagram of the bicistronic constructs. The construct pRF+EMCV contains the IRES of the encephalomyocarditis virus and was used as a positive control for IRES activity. (B) The constructs were transcribed and translated *in vitro* using T7 polymerase for transcription and a nuclease-treated rabbit reticulocyte lysate to translate the transcribed RNA. The Renilla and Firefly luciferase activities were determined after 80 minutes incubation. Firefly luciferase activities were normalized to Renilla luciferase levels. (C) Every 10 minutes 5 µl of the reaction was used to measure the control Renilla luciferase activity.

Again we did not observed any induction of the Firefly luciferase with the construct containing the *myeov* 5`UTR. In contrast, the construct containing the IRES of *EMCV* showed 10-fold induction over background pRF vector (Figure 3.10b). Cap dependent translation of the Renilla luciferase cistron (used as internal control) was positive for all three constructs tested in this *in vitro* assay (Figure 3.10c).

In this *in vitro* assay, we failed to proof IRES activity in *myeov* 5`UTR. One possible explanation could be that certain specific IRES-Trans-Activation Factors (ITAFs) required for IRES-dependent translation were not present in the rabbit reticulocyte lysate and therefore may explain our failure to show IRES activity of the *myeov* 5`UTR in this *in vitro* assay (Holcik et al., 2003; Kullmann et al., 2002; Millard et al., 2000; Stoneley et al., 2000).

## 3.7. IRES activity of the *myeov* 5`UTR during apoptosis

Several mRNAs whose proteins products are involved in apoptosis are translated by the alternative mechanism of internal ribosome entry (Hellen and Sarnow, 2001). Staurosporin is a strong inhibitor of protein kinase C and therefore this antibiotic has a potent toxic effect on several tumor cell lines, which in turn will drive these cells into apoptosis.

In order to verify whether the *myeov* putative IRES would be activated during apoptosis, we transiently transfected the DNAs from the constructs pGL3, pGL3+UTR, pRF and pRF+UTR into HEK 293 cells. Twenty four hours after transfection, 1 µM Staurosporin was added to the transfected cells, and twenty four hours after treatment with Staurosporin, cells were lysed and luciferase activities were measured (Figure 3.11a and b). Figure 3.11 shows the Firefly luciferase measured from cells transfected with the mono- and bicistronic constructs. Staurosporin drove about 30% of the cells into apoptosis, but no increase in Firefly luciferase activity could be demonstrated during treatment with Staurosporin.

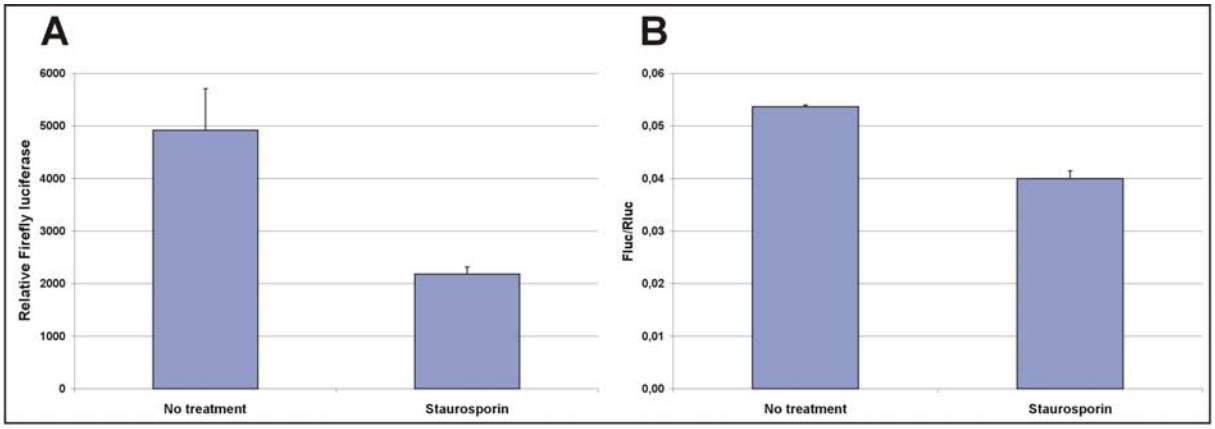



**Figure 3.11. The *myeov* putative IRES is not active during apoptosis.**
(A) HEK 293 cells were transfected with DNA of the monocistronic constructs pGL3+UTR and (B) with DNA of the bicistronic constructs pRF+UTR. Twenty four hours after transfection Staurosporin was added directly to the cells medium. Forty eight hours after transfection the cells were lysed and the activities of both reporter genes were measured. The empty vector pGL3 and pRF showed similar luciferases activities and are not depicted.

In addition to Staurosporin treatment, we also transfected cells with FADD (Fas-associated death domain containing molecule also called MORT1), and deprived cells of serum. FADD drives the activation of caspase 8, and this in turn leads to the cleavage of procaspase 3 and the triggering of the caspases cascade, ending in apoptosis (Zou et al., 1999). Deprivation of serum in the medium of cells also leads to apoptosis. Again we transfected 293 cells with pGL3, pGL3+UTR, pRF and pRF+UTR. In this experiment, we have divided the cells basically into four groups:

a) cells without treatment;
b) cells treated with Staurosporin;
c) cells transfected with FADD;
d) cells deprived of serum.

The cells in the first group (a) were lysed after 48 hours. The cells in second group (b) were treated with Staurosporin 24 hours after transfection of the luciferase reporter constructs, and lysed 24 hours after treatment. The cells in the third group (c) were transfected with FADD 24 hours after transfection of the luciferase reporter vectors, and lysed 12 and 24 hours after that. The cells in the fourth group (d) were deprived of serum 24 hours after transfection of the luciferase reporter constructs, and lysed 24 and 48 hours after that. The

luciferase measurements of the monocistronic and bicistronic constructs are shown in Figure 3.12a and b, respectively. Our analyses did not reveal IRES dependent activation by the *myeov* 5`UTR during apoptosis.

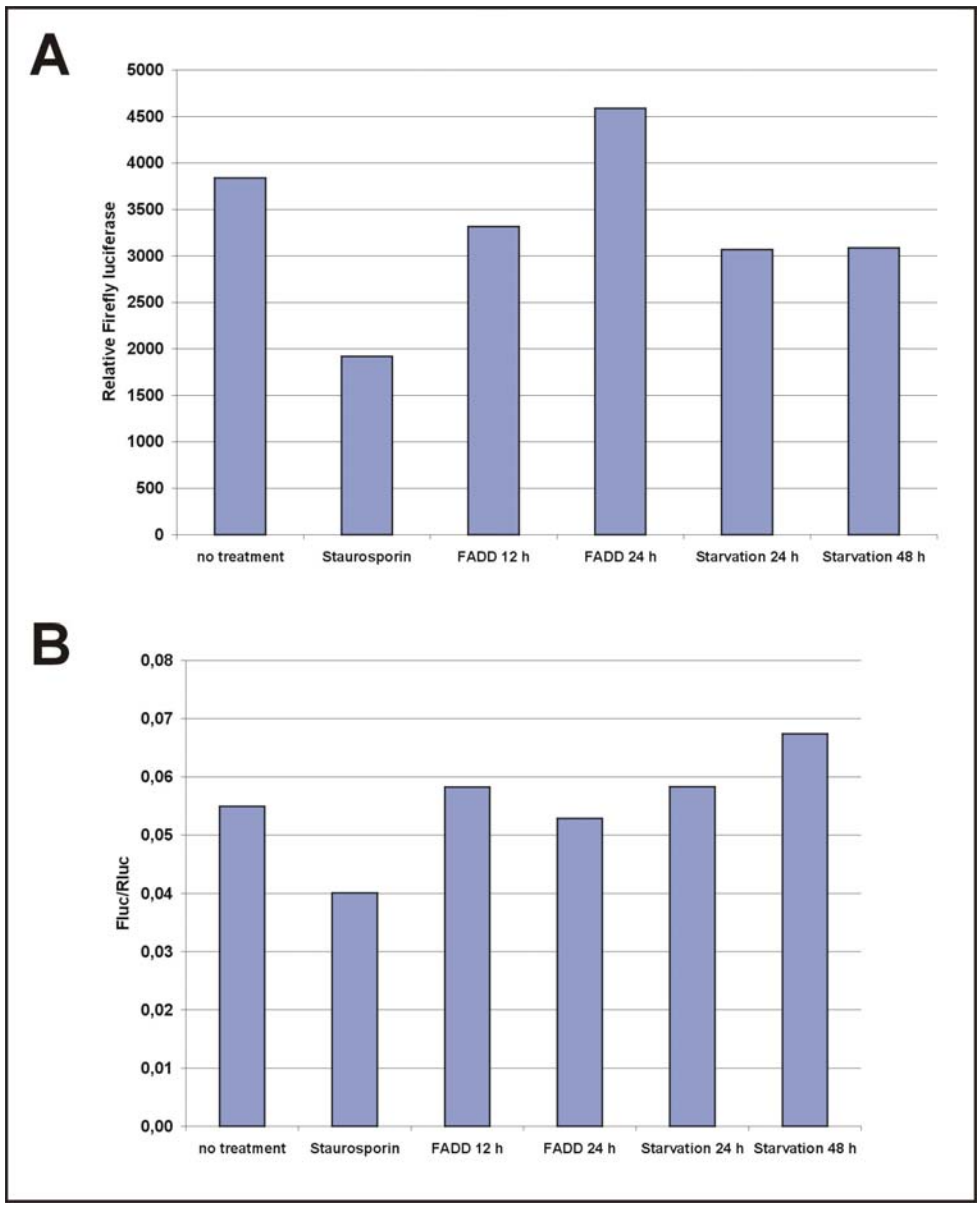



**Figure 3.12. No indication of *myeov* IRES activity during specific cell stress.**
(A) HEK 293 cells were transfected with DNA of the monocistronic construct pGL3+UTR and (B) with DNA of the bicistronic constructs pRF+UTR. Twenty four hours after transfection cells were stressed using different methods, such as: 1) the addition of Staurosporin to the cell's medium; 2) transfection with DNA of the adaptor molecule FADD (Fas-associated death domain-containing molecule) that induces apoptosis and 3) deprivation of the cells with serum for 24 and 48 hours. 72 hours after transfection the activities of both reporter genes were measured. The empty vector pGL3 and pRF showed similar luciferases activities and are not depicted.

## 3.8. Does *myeov* 5`UTR has a cryptic promoter?

In order to ensure that the Firefly luciferase activity observed in our bicistronic constructs was not due to the presence of a cryptic promoter in the *myeov* 5`UTR, we created a promoterless construct. For this purpose, we simply removed the SV40 promoter and the intron of the bicistronic plasmid vectors pRF, pRF+UTR and pRF+EMCV and religated the plasmid DNA, creating the vectors pRF-P, pRF-P+UTR and pRF-P+EMCV, respectively (Figure 3.13a). In this experiment no promoter is present in the constructs, and therefore no RNA transcription can occur and translation will be abrogated, and consequently no Firefly luciferase should be measured. The DNAs from the promoterless constructs were transiently co-transfected with the control plasmid pCMV-LacZ into 293 cells and luciferase activity was measured 48 hours after transfection. Firefly luciferase activity was normalized in pRF-P+UTR to the β-Gal activity. We observed a 10-fold higher luciferase activity compared to background (pRF-P). Surprisingly pRF-P+EMCV also showed 10-fold induction, suggesting a promoter activity in EMCV 5`UTR. (Figure 3.13b).

Despite the fact that we detected a 10-fold induction with both the *myeov* and EMCV 5`UTR constructs, the Firefly luciferase activity measured was rather lower. To ensure whether *myeov* 5`UTR harbors a cryptic promoter, we transfected again cells using the same set of constructs. These cells were lysed 48 hours after transfection, and mRNA was isolated, subjected to formaldehyde gel electrophoresis, transferred onto nitrocellulose membrane, and then hybridized with a $^{32}$P-labelled Firefly luciferase probe (Figure 3.13c). Our Northern Blot analysis showed a clear transcript and suggests the presence of a cryptic promoter in the *myeov* 5`UTR. We also observed a very faint band in RNA of cells transfected with pRF-P+EMCV, which may indicate the presence of promoter activity in the EMCV 5`UTR.

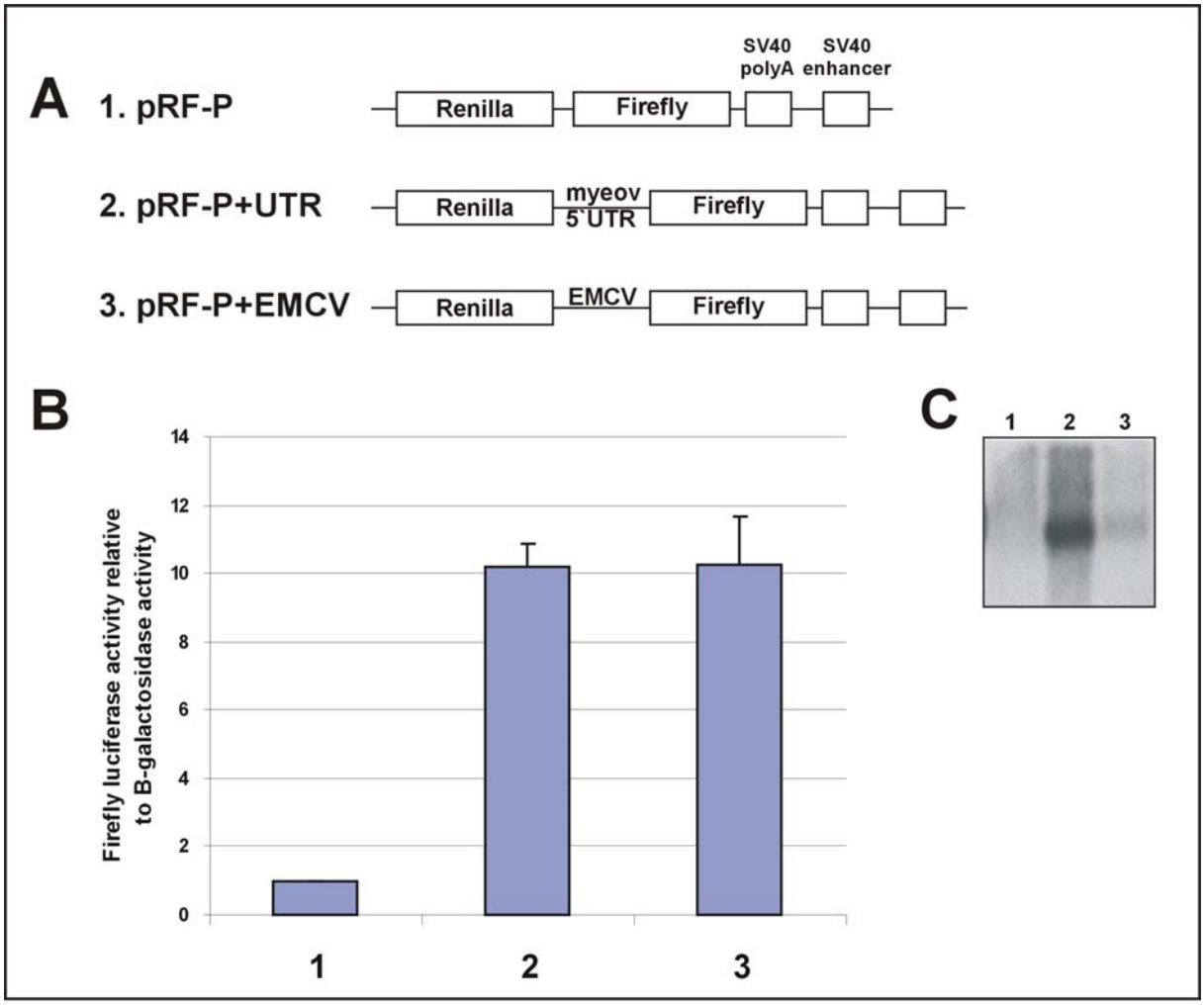



**Figure 3.13. Cryptic promoter activity of the *myeov* 5`UTR.**
(A) Schematic diagram of the promoterless bicistronic constructs pRF-P, pRF-P+UTR and pRF-P+EMCV. The sequence of the promoter and the chimeric intron were removed from the parenteral vector. (B) HEK 293 cells were transfected with DNAs of the promoterless constructs in combination with pCMV-LacZ, and 48 hours after transfection the activities of both reporter genes were measured. Firefly luciferase activity was 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. (C) Northern Blot analysis of poly($A^+$) mRNAs derived from transfected cells. The RNAs were electrophoresed in the presence of formaldehyde, transferred to nitrocellulose and then probed with a $^{32}$P-labelled Firefly luciferase fragment.

In order to check these data, we also removed the promoter of the monocistronic vectors pGL3+UTR and pGL3+EMCV, creating the constructs pGL3-P+UTR and pGL3-P+EMCV, respectively (Figure 3.14a). All these plasmid DNAs were transiently transfected into HEK 293 cells and 48 hours after transfection, one tenth of the cells were lysed and Firefly luciferase activity was measured (Figure 3.14b). The rest of the lysate was used to isolate mRNA.

Poly(A+) RNA was subjected to formaldehyde agarose gel electrophoresis, blotted onto nitrocellulose membrane, and hybridized with a $^{32}$P-labelled Firefly luciferase probe (Figure 3.14c).

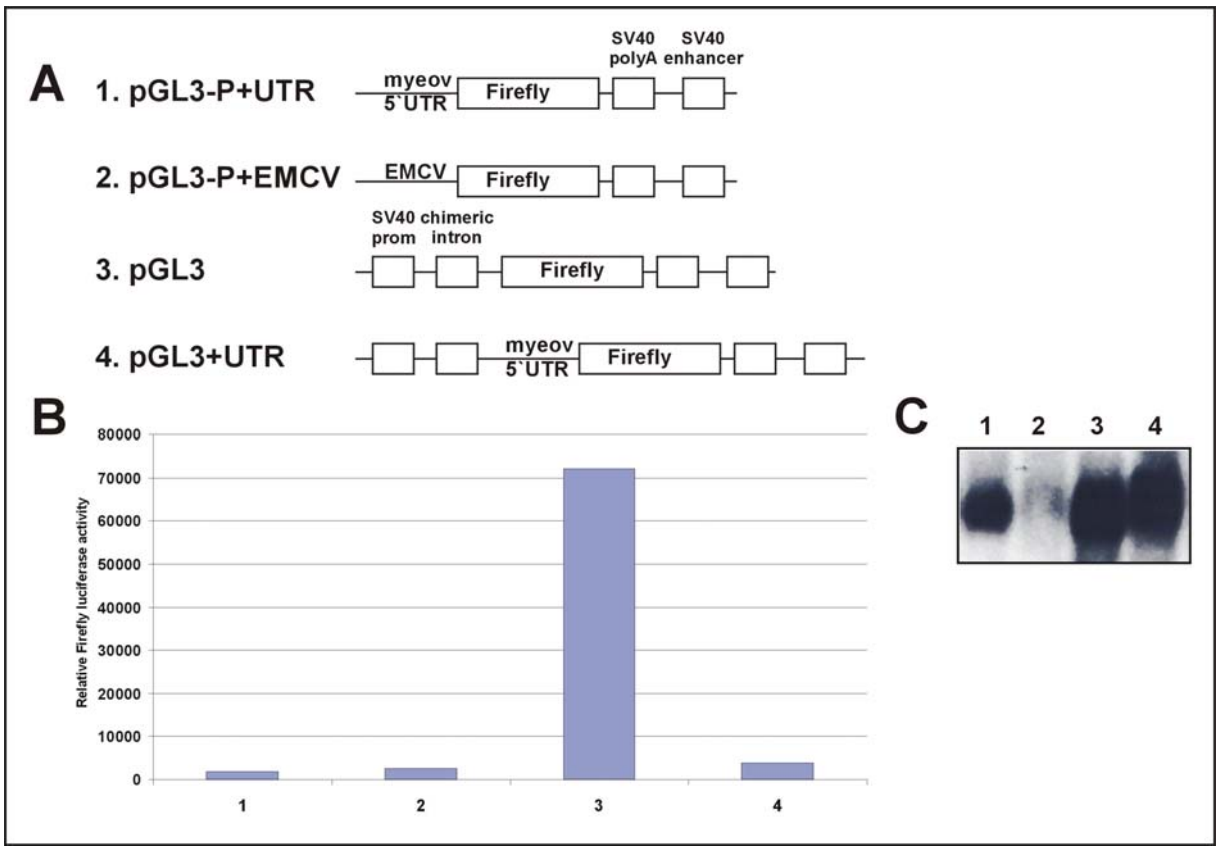



**Figure 3.14. Promoter activity of *myeov* 5`UTR in the promoterless monocistronic constructs.**
(A) Schematic diagram of the monocistronic constructs pGL3-P+UTR, pGL3-P+EMCV, pGL3 and pGL3+UTR. The promoter sequences were removed in the constructs pGL3-P+UTR (*myeov* 5`UTR) and pGL3-P+EMCV (EMCV 5`UTR). (B) HEK 293 cells were transfected with these constructs in combination with pCMV-LacZ, and 48 hours after transfection the activities of Firefly luciferase was measured. Firefly luciferase activity was normalized to β-galactosidase activity. (C) Northern Blot analysis of transfected cells using a $^{32}$P-labelled Firefly luciferase fragment.

The presence of a strong transcript in cells transfected with pGL3-P+UTR confirms the promoter activity of the *myeov* 5`UTR as observed in the bicistronic promoterless constructs. These data and the data from the previous experiment suggest the presence of a cryptic promoter in the *myeov* 5`UTR and no IRES. To confirm this result, we again transfected HEK 293 cells with DNAs of the following constructs: pRF, pRF+UTR (Figure 3.15a) and phpRF and phpRF+UTR (Figure 3.16a).

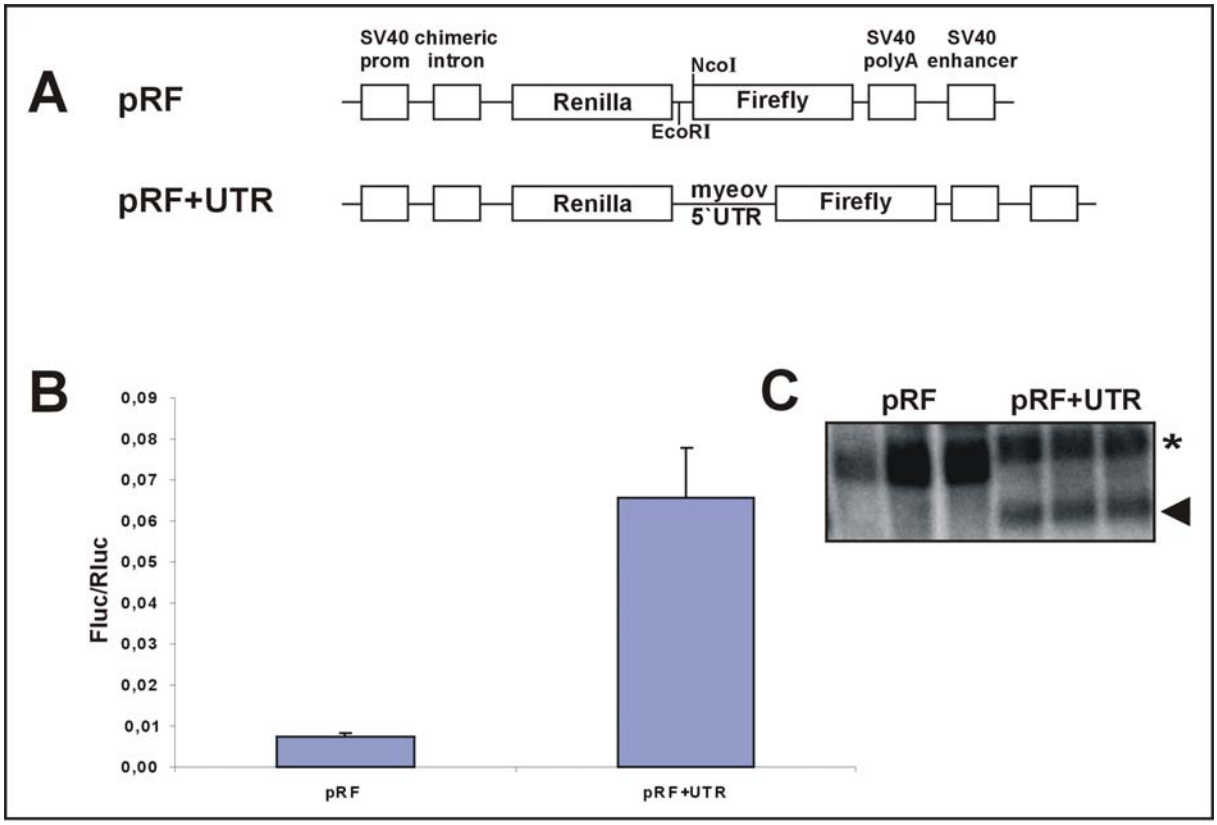



**Figure 3.15. Presence of promoter activity in the *myeov* 5`UTR.**
(A) Schematic diagram of the bicistronic constructs. *Myeov* 5`UTR was inserted using the indicated restriction sites into the intercistronic region. (B) HEK 293 cells were transfected with the described constructs and both luciferases activities were measured. Firefly luciferase was normalized to the internal control Renilla luciferase. (C) Northern Blot analysis of the mRNAs isolated from transfected cells, hybridized with a $^{32}$P-radiolabelled Firefly luciferase fragment. The asterisk indicates the bicistronic RNA that initiates at the SV40 promoter. The arrowhead indicates the position of the Firefly luciferase transcript that initiates in the *myeov* 5`UTR.

Forty eight hours after transfection, one tenth of the sample was used to measure the luciferases activity (Figure 3.15b and 3.16b). The rest of the lysate was used for mRNA extraction followed by formaldehyde gel electrophoresis, blotting and hybridization with a $^{32}$P-labelled Firefly luciferase probe (Figure 3.15c and 3.16c). If the observed Firefly luciferase was due to the presence of an IRES, one should be able to see only one transcript. However, if a promoter is present one should be able to see two transcripts. One transcript corresponding to the bicistronic RNA and one transcript corresponding to the monocistronic RNA produced by the *myeov* 5`UTR cryptic promoter. Figure 3.15c and 3.16c clearly show two transcripts: one long transcript corresponding to a bicistronic mRNA which is produced under the control of

the SV40 promoter, and one short mRNA corresponding to the promoter present in the *myeov* 5`UTR. This result was confirmed by hybridization of the same samples with a $^{32}$P-labelled Renilla luciferase probe, where only the long transcript was observed (data not shown).

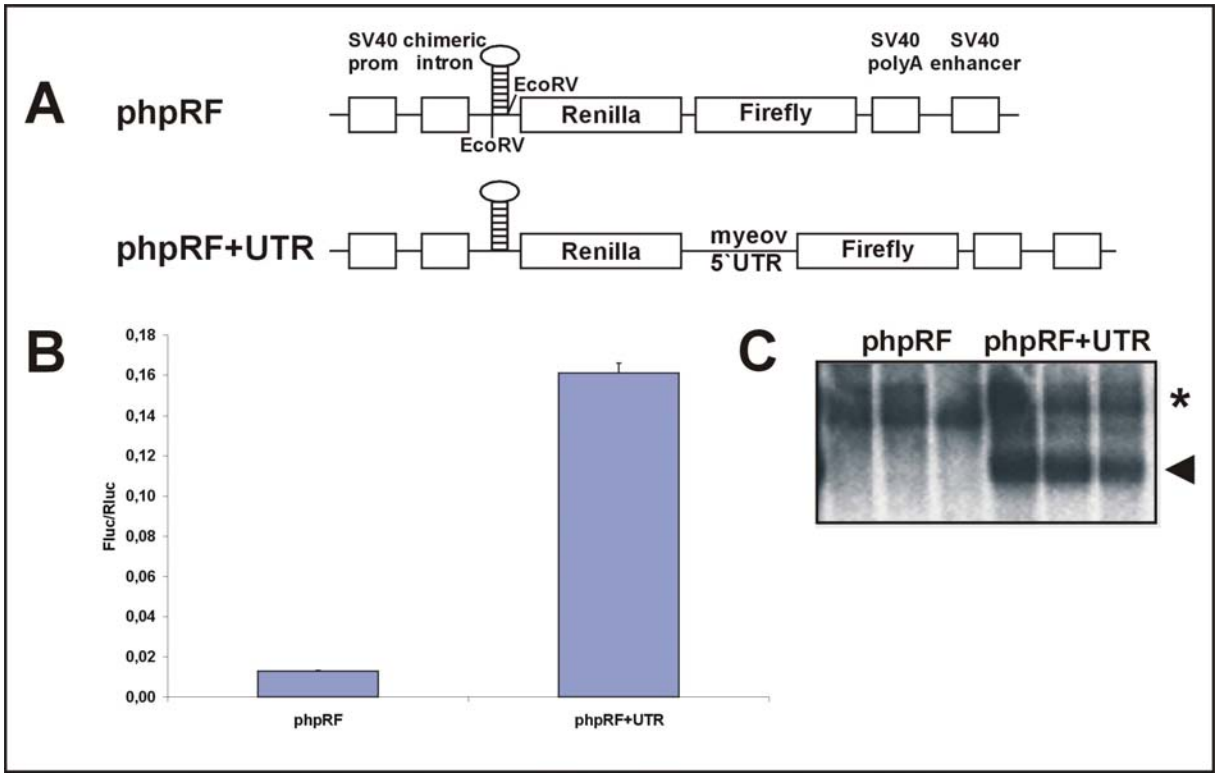



**Figure 3.16. Presence of promoter activity in the *myeov* 5`UTR using the bicistronic construct with a stable hairpin.**
(A) Schematic diagram of the bicistronic constructs. A stable hairpin (-55 kcal/mol) was inserted in the indicated restriction site upstream of the Renilla luciferase cistron. (B) HEK 293 cells were transfected with these constructs and the luciferases activities were measured. (C) Northern Blot analysis of the RNAs isolated from transfected cells, hybridized with a $^{32}$P-labelled Firefly luciferase fragment. The asterisk indicates the bicistronic RNA that initiates at the SV40 promoter. The arrowhead indicates the position of the Firefly luciferase transcript that initiates in the *myeov* 5`UTR.

## 3.9. Mapping the *myeov* 5`UTR promoter

In order to determine the precise location of *myeov* promoter activity in its 5`UTR, we generated several promoter deletions constructs as shown in Figure 3.17a. The advantage to use the bicistronic vectors containing the SV40 promoter is that the bicistronic mRNA is transcribed and the Renilla luciferase activity (translated via a cap-dependent mechanism) can be used

to measure transfection efficiency. The constructs were transfected into HEK293 cells and 48 hours after transfection the cells were used to measure luciferase activity (Figure 3.17b).

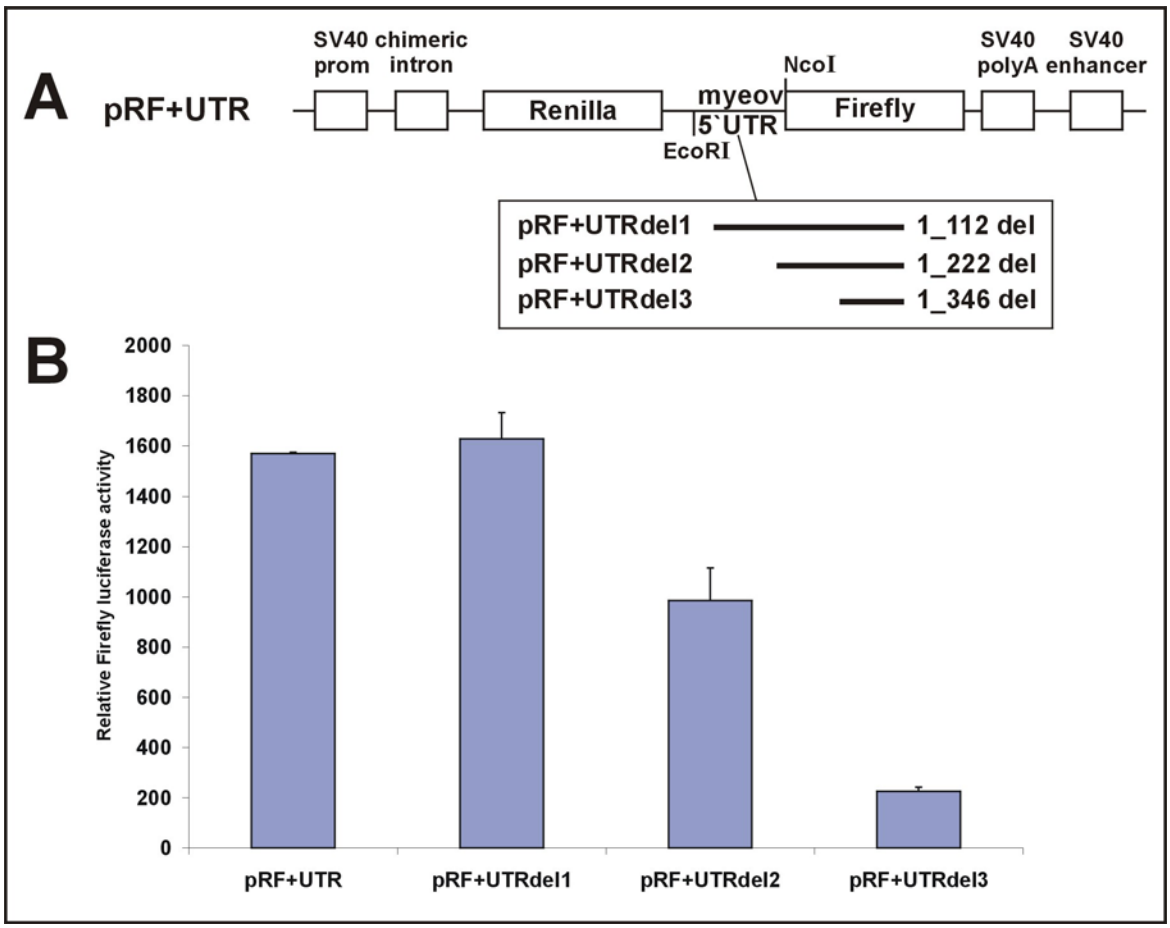



**Figure 3.17. Deletion mapping of the *myeov* cryptic promoter.**
(A) Deletions of the *myeov* 5`UTR were created by PCR, and the deletion products were introduced into the bicistronic vector pRF, creating the constructs pRF+UTRdel1, pRF+UTRdel2 and pRF+UTRdel3. (B) HEK 293 cells were transiently transfected with these constructs and both luciferases activities were measured.

As demonstrated by the Firefly luciferase activity (Figure 3. 17b) we could see the higher Firefly luciferase levels in cells transfected with the first two constructs (*myeov* 5`UTR wild type and the construct in which 112 nucleotides from the 5`end were removed "deletion 1"). In cells transfected with DNA from the last two constructs (deletion 2 and 3, in which 222 and 336 nucleotides from the 5` end of the *myeov* 5`UTR were removed) the level of the Firefly luciferase was reduced. This suggests that the *myeov* promoter region is located between nucleotides 113 and 223 (Figure 3.18).

The results obtained so far clearly demonstrated the presence of a cryptic promoter in the *myeov* 5`UTR. The intensity of the transcript originating from the *myeov* 5`UTR promoter was similar to that of the one produced by the SV40 promoter (see Figure 3.15c and 3.16c, please compare the upper band (bicistronic RNA) and the lower band (monocistronic) RNA). This result implies that the *myeov* 5`UTR promoter is a very efficient promoter almost comparable to the strong SV40 promoter.

Despite high transcription levels, translation of Firefly luciferase was strongly impaired in the monocistronic as well as the bicistronic constructs when compared to the Renilla luciferase activity translated from the transcript originating from the SV40 promoter. In order to clarify this discrepancy, we decided to investigate whether the uAUGs present in the *myeov* 5`UTR could be responsible for the reduced protein level produced by our luciferase reporter plasmids.

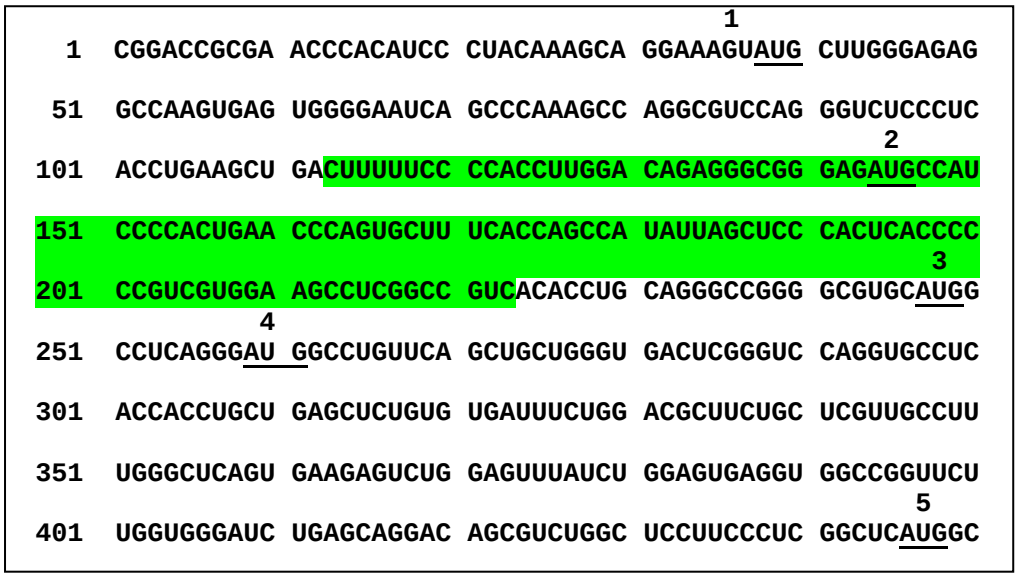



**Figure 3.18. Mapping of *myeov* 5`UTR promoter activity.**
The possible *myeov* sequence responsible for its cryptic promoter activity is shown in green. The expected transcript from the *myeov* cryptic promoter encompasses two or three upstream open reading frames. The start codons present in the *myeov* 5`UTR are underlined. The AUG number 5 indicates the *myeov* start codon that codes for the MYEOV protein.

## 3.10. Regulation of translation efficiency by the *myeov* 5`UTR

Despite strong promoter activity found in the *myeov* 5`UTR, translation of the Firefly luciferase cistron was much lower than that of the Renilla luciferase cistron, when using the bicistronic construct. These results suggest that, albeit strong Firefly luciferase transcription from the cryptic promoter in the *myeov* 5`UTR, translation of this transcript was severely blocked. For this reason, we decided to evaluate the effect of the four uAUGs present in the *myeov* 5`UTR on translation of the reporter gene. We therefore mutated the uAUGs of the *myeov* 5`UTR to AAG using the mutagenesis kit from Stratagene. For this purpose, *myeov* 5`UTR DNA was amplified using the primers myeovUTRHindIIIfor and myeovEcoRVrev, containing HindIII and EcoRV restriction sites. The PCR fragment was cloned into pGEM-T easy vector, creating the vector pGEM-T+myeov5`UTRHindIIIEcoRV, checked by restriction digestion and sequencing. Subsequently this construct was used as a template for mutagenesis. Mutagenesis was performed using four primers (myeovATG1, myeovATG2, myeov ATG3, and myeovATG4). Each primer was designed to make a mutation of the uracil nucleotide in the start codon of the uORF, where U was replaced to A. In this way the AUG start codon was replaced by the codon AAG, which codes for leucine. The mutated fragment was checked by sequencing and subcloned upstream of Renilla luciferase cistron into the plasmid vector pRF+EMCV, thus creating the vector pRF+EMCV+myeov1,2,3,4mut. The wild type *myeov* 5`UTR fragment without mutations was also cloned into pRF+ECMV to create the vector pRF+EMCV+myeov without mutation (Figure 3.19a). In these constructs the fist cistron is translated via a cap dependent mechanism and the second cistron is cap-independently translated via the IRES of EMCV. The Firefly luciferase activity produced is used as an internal control to normalize for transfection variations. The DNA constructs were transfected into HEK 293 cells, and 48 hours after transfection cells were lysed and luciferase activities were measured (Figure 3.19b). The corrected Renilla luciferase activity was high in the empty vector (pRF+EMCV), where Renilla luciferase was translated via a cap-dependent mechanism. In the construct containing the *myeov* 5`UTR

lacking any mutation (pRF+EMCV+myeov without mutation). Renilla luciferase activity was strongly reduced (96%). This result was in agreement with previous observations, where the *myeov* 5`UTR has been shown to block protein translation. Transfection of pRF+EMCV+myeov1,2,3,4mut in which all uAUGs were removed, almost completely restored the level of Renilla luciferase. These results clearly show that upstream AUG triplets in the *myeov* 5`UTR transcript can regulate the expression on the level of translation.

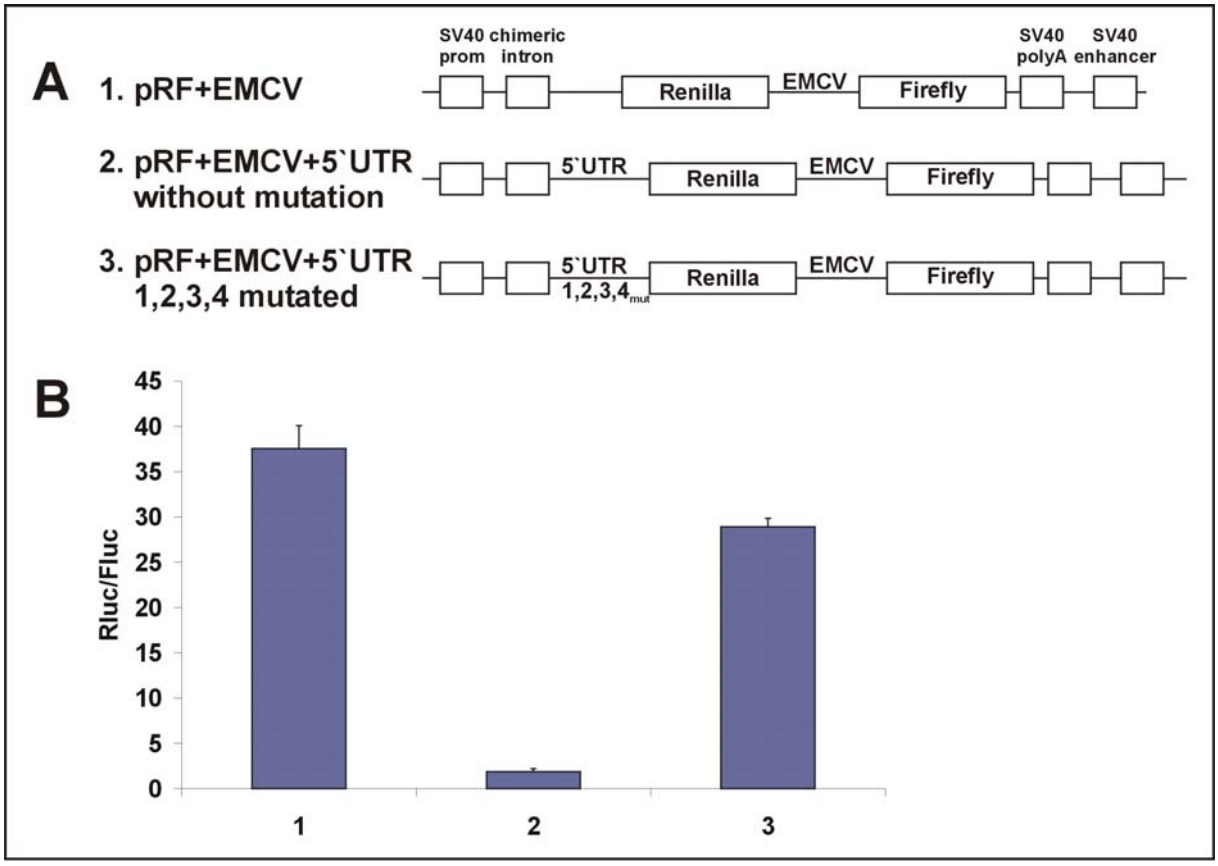



**Figure 3.19. Effect of *myeov* 5`UTR wt or mutated on translation of reporter gene.**
(A) Schematic diagram of the bicistronic reporter vector pRF+EMCV. In these constructs the translation of the Firefly luciferase cistron is mediated by the EMCV internal ribosome entry site (or cryptic promoter). The *myeov* 5`UTR wild type or the mutated form, where the four AUG start codon of the *myeov* 5`UTR was mutated to AAG, were inserted upstream of the first Renilla cistron (pRF+EMCV+5`UTR without mutation and pRF+EMCV+5`UTR 1,2,3,4 mutated, respectively). (B) HEK 293 cells were transfected with these constructs and both luciferases activities were measured. The Renilla luciferase activity was normalized to the internal control Firefly luciferase.

In order to evaluate the contribution of individual uAUGs to the repression of translation of the reporter gene constructs several constructs with single and/or combinations of AUG-AAG mutations were designed. For this purpose we used pGEM-T+myeov5`UTRHindIIIEcoRV as a template and the mutagenic primers myeovATG1, myeovATG2, myeovATG3 and myeovATG4 and the mutagenesis kit. Several mutational combinations were constructed. The mutated plasmid DNAs were transformed into bacteria, mini-preparation was performed, and DNAs were sequenced. After having sequenced many miniprep DNAs we finally ended up with a complete set of constructs representing all possible mutational combinations (in total 16 constructs containing various mutations, and one wild type). The *myeov* 5`UTR cloned into T-vector was subcloned into the vector pRF-EMCV upstream of the Renilla luciferase reporter gene, creating pRF+EMCV+UTRmutated (Figure 3.20a). All constructs were transiently transfected into HEK 293 cells and the luciferase activities were measured 48 hours after transfection (Figure 3.20b). As depicted in Figure 3.20b, all mutations had some effect on the translation efficiency, but a clear preference could not be observed.

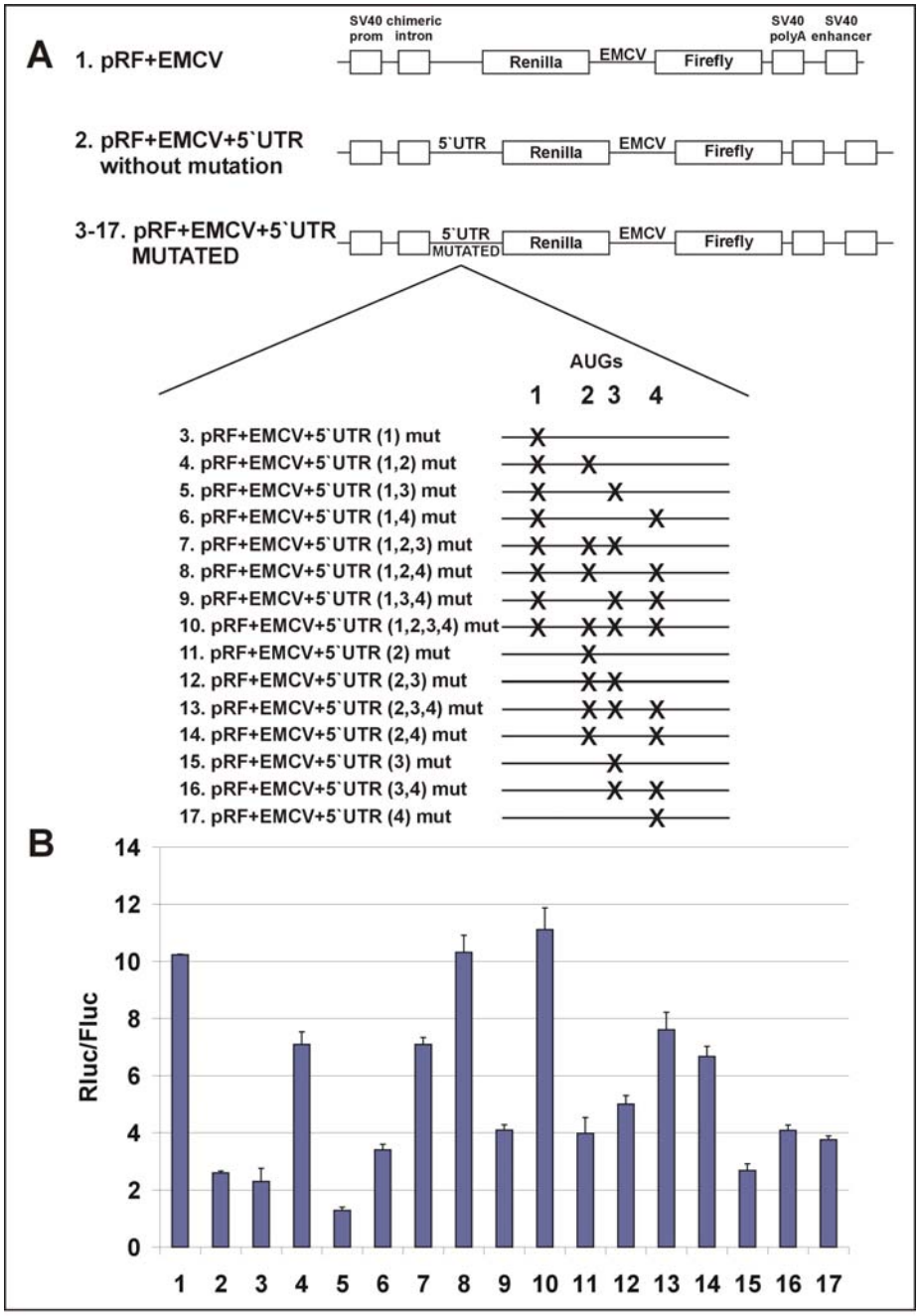



**Figure 3.20. Effect of different *myeov* 5`UTR upstream start codon mutation on translation of the reporter gene.**
(A) Schematic diagram of the bicistronic vector pRF+EMCV. The *myeov* 5`UTR wild type was inserted upstream of the Renilla luciferase cistron. The myeov AUGs were mutated to AAG. The respective mutations are indicated by an "X" in the constructs 3 to 17. (B) HEK 293 cells were transfected with the wild type or the mutant constructs and luciferases activities were measured. Renilla luciferase was normalized to the internal control Firefly luciferase. The translation of the Firefly luciferase reporter gene is mediated by an IRES of the encephalomyocarditis virus.

## 3.11. RNA transfection

In order to analyze the effect of uAUGs in the myeov 5`UTR directly, without the interference of possible transcriptional effects due to, for example, promoter competition for transcription factors, we transiently transfected *in vitro* transcribed RNAs into HEK 293 cells. For this experiment, we used the bluescript based vector containing a T3 promoter upstream of the Firefly luciferase reporter gene. *Myeov* 5`UTR wild type or the mutated *myeov* 5`UTR, in which all four uAUGs were mutated to AAG were amplified by PCR and SpeI and NcoI restriction sites were added. The purified PCR fragments were cloned into pGEM-T easy vector, originating the vector pGEM-T+myeovSpeINcoI and pGEM-T+myeovSpeINcoI1,2,3,4mut, respectively. Clones were verified by sequencing and the *myeov* 5`UTR fragments were subcloned into pBSK, creating the vectors pBSK+UTR and pBSK+UTR1,2,3,4mut (Figure 3.21a). The T3 promoter present in these vectors was used for *in vitro* transcription using T3 polymerase. Prior to *in vitro* transcription the constructs were linearized with the restriction enzyme XhoI, that is located downstream of the Firefly luciferase reporter cistron. In this way we obtained one transcript of the *myeov* 5`UTR (wild type and mutated) and the reporter gene Firefly luciferase. The DNA fragment was purified and synthetic mRNA was generated using the mMESSAGE mMachine™ reaction system (Ambion) as described in Materials and Methods. The transcribed mRNA was purified and quantitated by spectrophotometer analysis and agarose gel electrophoresis. RNAs were transfected into HEK 293 cells. Twenty four hours after transfection, cells were lysed and part of the lysate was used to measure the Firefly luciferase activity (Figure 3.21b), and the rest of the lysate was used to extract mRNA. The RNAs were applied to a formaldehyde agarose gel, blotted and hybridized with a $^{32}$P-labelled Firefly luciferase probe and quantitated by phosphoimager (Figure 3.21c). As we can see in Figure 3.21b, the high Firefly luciferase activity in pBSK was abolished when myeov 5`UTR wild type was placed upstream of the Firefly luciferase cistron. Firefly luciferase activity levels

were restored to a normal level when we replaced the myeov 5`UTR for a mutated form of the myeov 5`UTR.

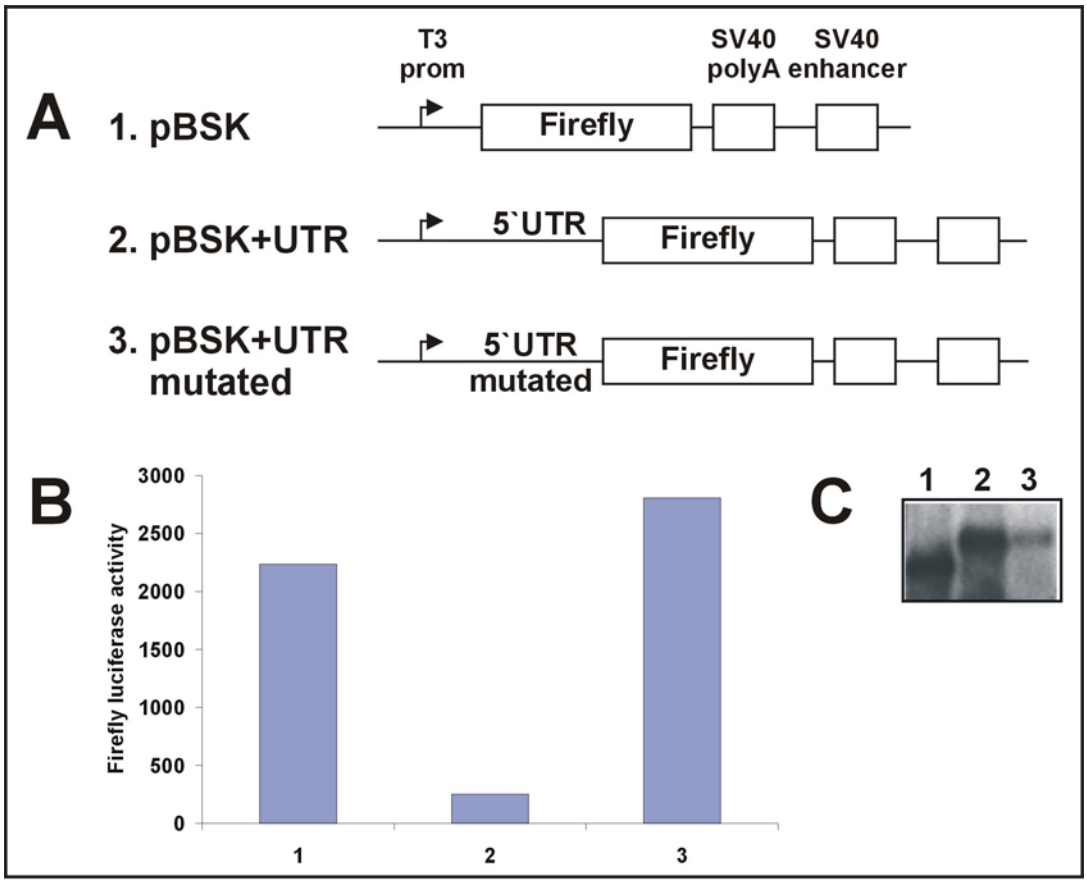



**Figure 3.21. Effect of the myeov upstream open reading frame on translation of the reporter gene by RNA transfection.**
(A) Schematic diagram of the DNA constructs pBSK, pBSK+UTR (wild type) and pBSK+UTRmutated, in which all four uAUGs were mutated to AAG. The DNA constructs were linearized and transcribed *in vitro* using the T3 promoter. (B) HEK 293 cells were transiently transfected with the purified RNAs transcribed *in vitro*, and 24 hours after transfection the Firefly luciferase activities were measured. (C) Poly($A^+$) RNAs were isolated from the transfected cells, electrophoresed in the presence of formaldehyde, transferred to nitrocellulose membrane and then probed with a $^{32}P$-radiolabelled Firefly luciferase fragment.

This result was in agreement with our DNA transfection data and shows once more that the myeov uAUGs are responsible for the control of protein translation of the reporter gene.

## 3.12. Is the *myeov* upstream open reading frame responsible for MYEOV protein translation control?

Our previously mentioned data strongly suggest that *myeov* uAUGs are responsible for protein translation control, since the wild type *myeov* 5`UTR can drastically abolish protein translation, and a mutated form of the *myeov* 5`UTR restores normal protein levels as shown in our DNA and RNA transfection experiments. Although these results clearly show that uAUGs can control protein translation of reporter genes, we were interested to check whether the same effect could be observed using the complete *myeov* cDNA sequence. Therefore, we cloned the complete wild type *myeov* cDNA sequence, and a cDNA sequence in which all uAUGs in the 5`UTR were mutated and sequences that were missing either the 5`UTR, the 3`UTR or both into the eukaryotic expression vector pMT2SM (Fig. 3.22a). Constructs were transiently transfected into HEK 293 cells and 48 hours after transfection RNAs and proteins were isolated. The RNAs were submitted to formaldehyde agarose electrophoresis, blotted and probed with $^{32}$P-labelled *myeov* (Figure 3.22b). Proteins were subjected to 10% SDS-PAGE, blotted and incubated with MYEOV specific antibodies (Figure 3.22c). Northern blot analysis showed specific transcripts of the expected sizes. The first lane of the SDS-PAGE gel shows proteins derived from transfection of the construct in which the start codon of the main *myeov* ORF was altered into an optimal Kozak start codon. Only one MYEOV specific protein band corresponding to the large MYEOV protein of 313 amino acids could be seen. Lane two shows the results after transfection with the *myeov* ORF with its own AUG. Two bands could be observed by Western Immunoblotting analysis. The two proteins are probably deduced from the long ORF of 313 amino acids 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 acids. Lanes three and four in the Northern Blot analysis show a very strong transcript, which corresponds to the complete *myeov* cDNA, however no protein could be detected (compare lanes three and four of the Northern Blot with the Western blot).

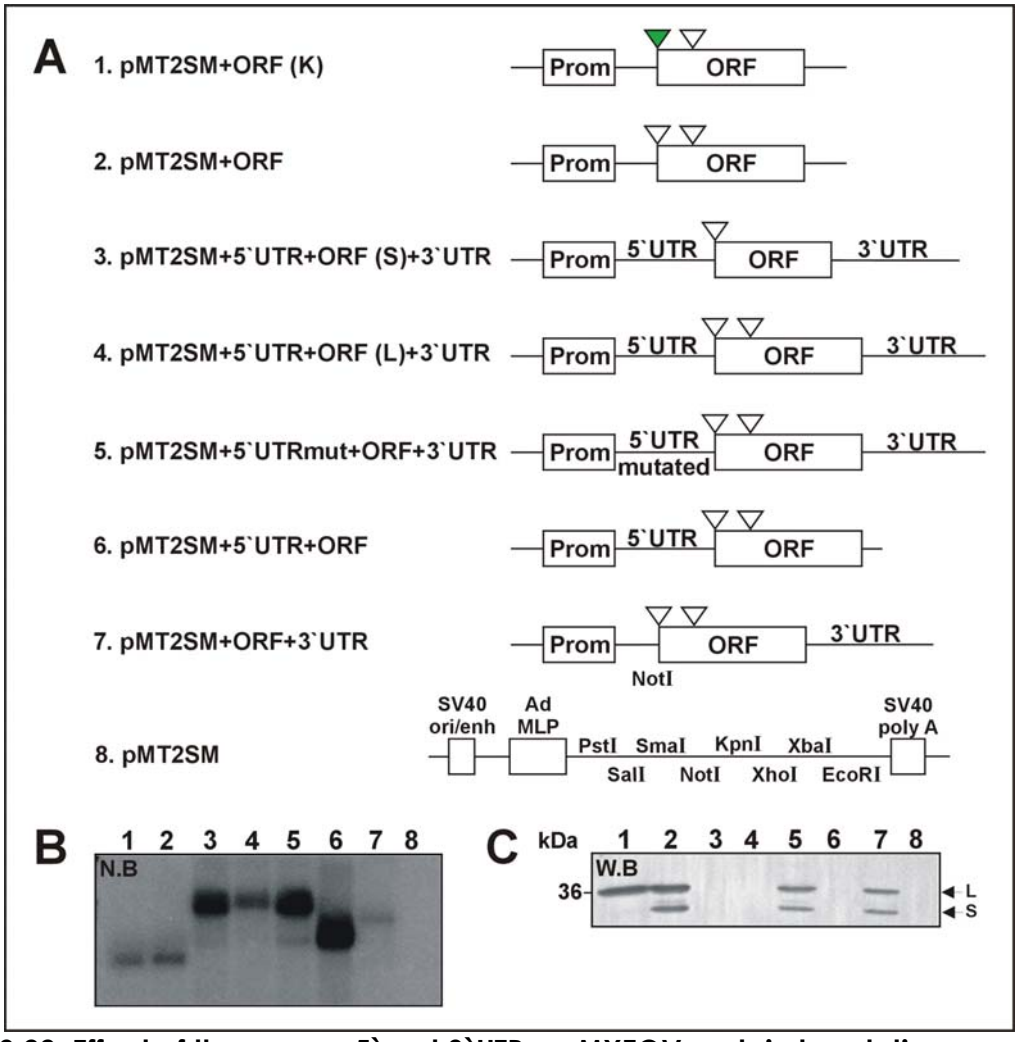



**Figure 3.22. Effect of the *myeov* 5`and 3`UTR on MYEOV protein translation.**
(A) Schematic diagram of the different *myeov* constructs in the eukaryotic expression vector pMT2SM (vector, number 8). Different combinations of fragments or mutated fragments of the complete *myeov* cDNA were inserted downstream of the adenovirus promoter of the pMT2SM vector. Filled green 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) HEK 293 cells were transfected with these cDNA constructs and 48 hours after transfection cells were harvested for RNA and protein extraction. RNAs were analyzed by Northern Blot analysis using a specific 5`*myeov* (position 1-894) probe and (C) proteins were analyzed by Western blotting using MYEOV-specific antibody. Positions of MYEOV proteins derived from the short (S) and long (L) open reading frame.

Lane five shows that mutation of the uAUGs in the *myeov* 5`UTR restores translation of MYEOV in the construct containing the complete *myeov* cDNA. Lane 6 shows the results of the transfection with the construct lacking the *myeov* 3`UTR. Although a transcript can be detected, no MYEOV protein could be seen, demonstrating that the *myeov* 3`UTR had no obvious inhibitory effect on MYEOV protein translation. Different results were obtained when the

5`UTR was removed (lane 7). Although the level of *myeov* RNA expression was rather low, clear MYEOV proteins could be observed on the Western blot. In summary these results strongly suggest that *myeov* uAUGs can regulate MYEOV biosynthesis on the level of translation and *myeov* uAUGs are responsible for a strong reduction of MYEOV protein translation.

### 3.1.3. Can MYEOV function as a transcription factor?

To answer this question, we have performed one experiment based on the knowledge that a transcription factor consists of two domains: a binding domain (BD) and an activating domain (AD). Two plasmid vectors were used to perform this experiment, namely pBIND and pG5luc. The vector pBIND has two cistrons: the first cistron contains GAL4-BD under the control of a CMV promoter, and in the second cistron the reporter gene Renilla luciferase is under the control of a SV40 promoter, and is used as transfection control. The monocistronic vector pG5luc has the reporter gene Firefly luciferase under the control of the adenovirus promoter, which is located downstream of five GAL4- DNA binding site. This implies that in order to start transcription, a GAL4-transcription factor (containing BD and AD) should bind to the GAL4- DNA binding sites, recruit the transcriptional machinery and drive transcription and translation of the Firefly luciferase reporter gene. In a situation where the AD of the transcription factor has been removed, the transcription factor is still able to bind to the binding sites in the DNA. However, as the AD is missing, transcription can not start.

To verify whether MYEOV codes for a transcription factor, we amplified the *myeov* ORF (coding for a long protein of 313 amino acids) and the AD of the transcription factor VP16 as a control. BamHI and XbaI restriction sites were added by PCR, and the purified fragments were cloned into the vector pGEM-T, creating pGEM-T+myeovORFBamXbaI and pGEM-T+VP16BamXbaI, respectively. The fragments were checked by sequencing and subcloned into pBIND in frame with the Gal4-Binding Domain (GAL4-BD), creating the

constructs pBIND+myeov and pBIND+VP16. Three different transient transfections into HEK 293 cells were performed (Figure 3.23):

a) pBIND and pG5luc (negative control);
b) pBIND+VP16 and pG5luc (positive control);
c) pBIND+myeov and pG5luc

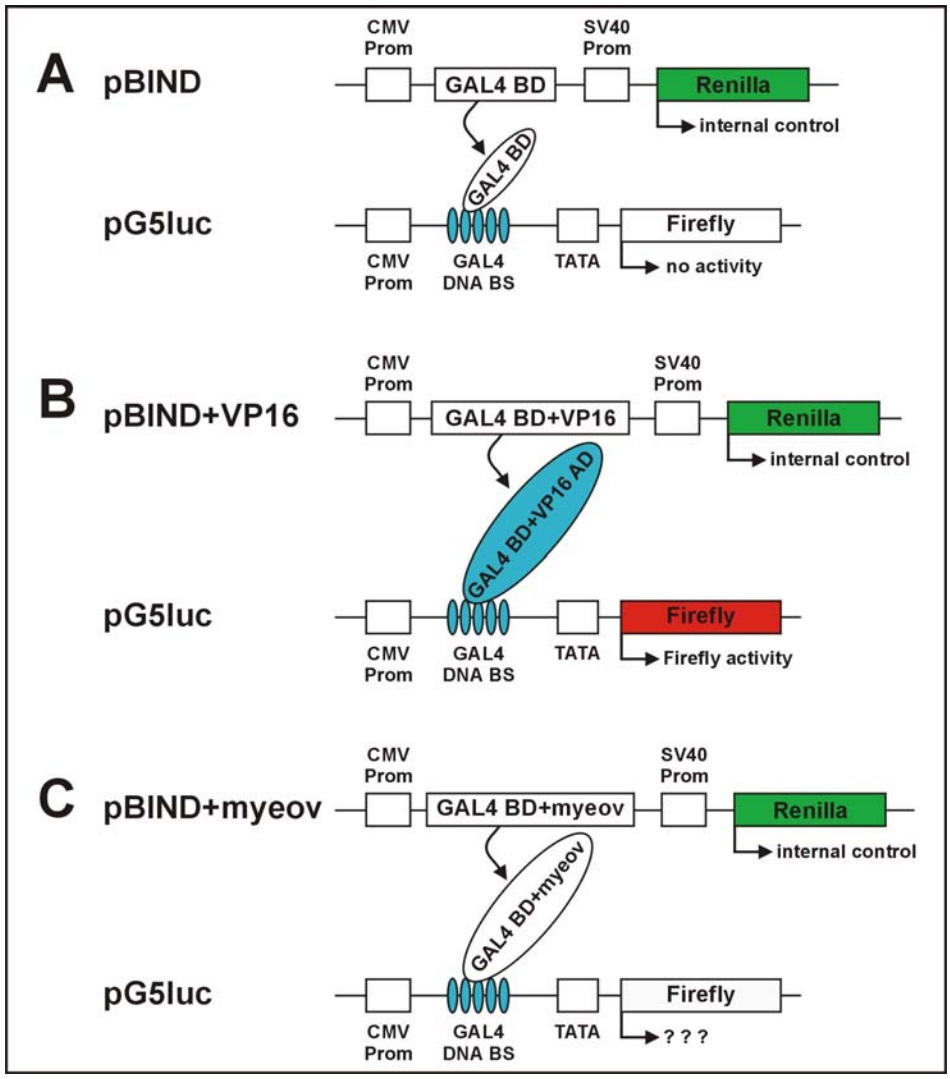



**Figure 3.23. Transcription factor constructs.**
(A) The plasmid pBIND contains the GAL4-BD under control of a CMV promoter. The Renilla luciferase is under control of the SV40 promoter and is used as internal control. The reporter plasmid pG5luc contains five GAL4-DNA binding sites. The Firefly luciferase reporter gene is used to measure transcriptional activation. (B) The activation domain of the transcription factor VP16 was inserted in frame with GAL4-BD. In this situation, a reconstituted transcription factor consisting of the GAL4 binding domain and the activation domain of VP16 binds to the GAL4-DNA binding sites and recruits the transcriptional machinery, resulting in transcription and translation of the reporter Firefly luciferase. (C) The *myeov* long open reading frame was inserted in frame with the GAL4-BD. However this fusion protein was not able to activate transcription of the reporter construct.

Forty eight hours after transfection the Luciferases activities were measured (Figure 3.24a). In the first transfection the GAL4-BD vector lacks the AD. GAL4-BD can bind to the GAL4 DNA binding sites in pG5luc, but transcription is not activated and as consequence no translation of the reporter gene Firefly luciferase occurs. In the second transfection the pBIND vector contains the AD of VP16 fused in frame with the GAL4-BD. The GAL4-BD/VP16AD fusion protein can bind to the GAL4 binding sites on the DNA and activate transcription. In the third transfection we did not detect any Firefly luciferase activity, suggesting that MYEOV is not able to activate transcription. In all transfections the Renilla luciferase activity of the pBIND vector was used as a transfection control. In addition we also decided to perform a Western Immunoblot analysis with GAL4-BD and MYEOV specific antibodies to check for proper expression of the transfected DNAs (Figure 3.24b). Proteins of expected sized were detected.

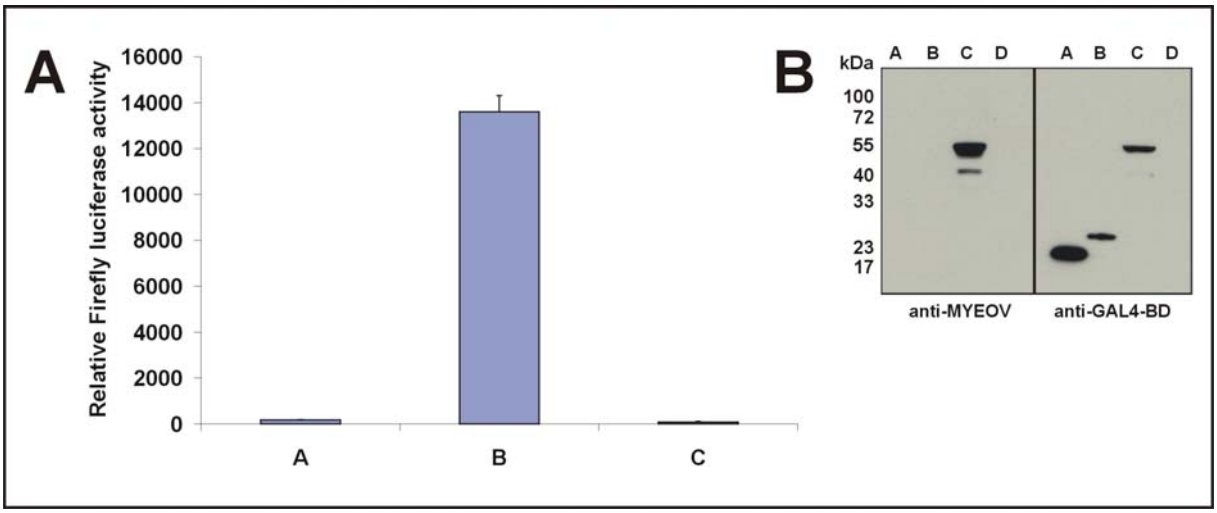



**Figure 3.24. *Myeov* is not a transcription factor.**
(A) The DNA constructs depicted in Figure 3.23 were transiently transfected into HEK 293 cells and forty-eight hours after transfection the activity of both reporter genes were measured. (B) Proteins from transfected cells were isolated and submitted to Western immunoblotting and incubated with either MYEOV specific antibody or GAL4-BD antibody. Fragments of expected size were detected. Co-transfections: A = pBIND and pG5-luc; B = pBIND-VP16AD and pG5luc; C = pBIND+myeov and pG5luc; D = untransfected cells.

The same set of plasmid DNAs were also transfected into another cell line, Met-5A. Met-5A expresses a SV40 large T antigen, which promotes replication from the SV40 origin found in the pBIND vector. The combination of large T antigen and SV40 origin may result in a higher copy number of this vector in the cells, and therefore increase expression of the reporter gene. As expected, the Renilla luciferase measured in the lysate extracted from Met-5A cells was about 10-fold higher compared to lysates from HEK 293 cells (Figure 3.25). However also in these cells Firefly luciferase was not activated when pBIND-myeov was used, implying that MYEOV could not activate transcription in Met-5A cells as well as in 293 cells. In conclusion these data show that MYEOV does not act as a transcription factor.

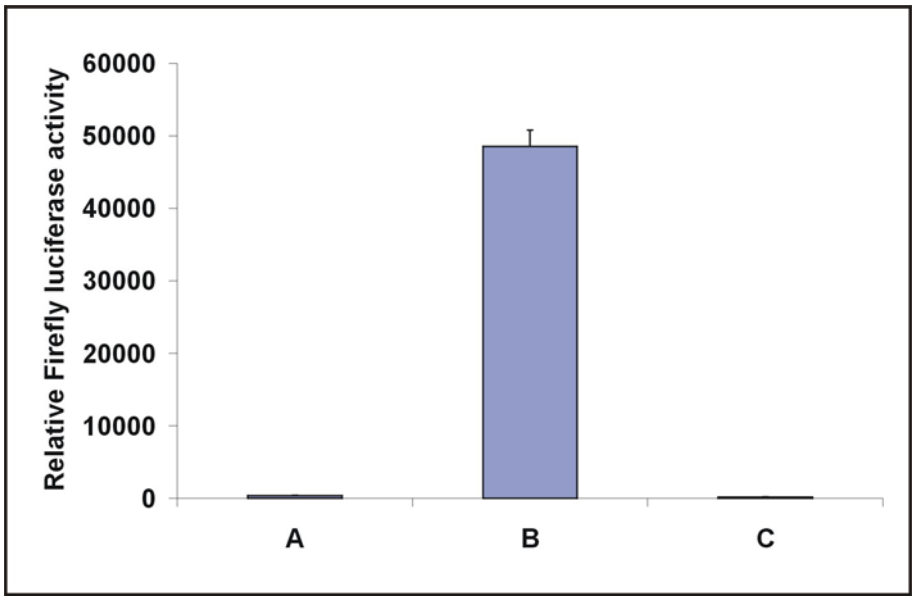



**Figure 3.25. *Myeov* does not act as a transcription factor in Met-5A cells.**
The DNA constructs depicted in Figure 3.23 were transfected into Met-5A cells. Forty-eight hours after transfection the activities of both reporter genes were measured. Co-transfections: A = pBIND and pG5-luc; B = pBIND-VP16AD and pG5luc; C = pBIND+myeov and pG5luc.

## 3.1.4. MYEOV protein in adenocarcinoma cell lines

In a previous study we reported high RNA levels of *myeov* in different esophageal squamous cell carcinoma cell lines (Janssen et al., 2002). Next we wanted to find out whether MYEOV protein is expressed in human

carcinoma cell lines, and if yes, whether those cells possibly contain alternative transcripts in which *myeov* uAUGs might be not present. Thirty samples from patients suffering of esophageal squamous cell carcinomas were donated by Dr. Yutaka Shimada (Department of Surgery & Surgical Basic Science, Kyoto University Hospital, Japan) and were analyzed by Western Immunoblotting. Unfortunately we failed to detect any MYEOV protein in all samples tested (data not shown). In previous studies, we have seen anomalous *myeov* transcripts in pancreas tissue and we therefore decided to check whether pancreas adenocarcinoma cell lines might express MYEOV protein. We used the pancreas cell lines Capan 1 and Dan-G. In addition, other cell lines were used: Met-5A, Hep-G2, HEK-293 and COS-7. Cells were cultivated as described in Materials and Methods and mRNA and proteins were isolated for Northern and Western Immunoblotting analysis, respectively (Figure 3.26a and b). The results of the Western blot analysis revealed endogenous MYEOV protein in Dan-G and COS-7 cells. We know from a previous study that KMS-12 show high *myeov* RNA levels that are not translated. Our Northern blot analysis shows high *myeov* transcript levels in Capan-1 also express *myeov*, but no MYEOV protein could be detected in the Western blot. This point has to be clarified further.

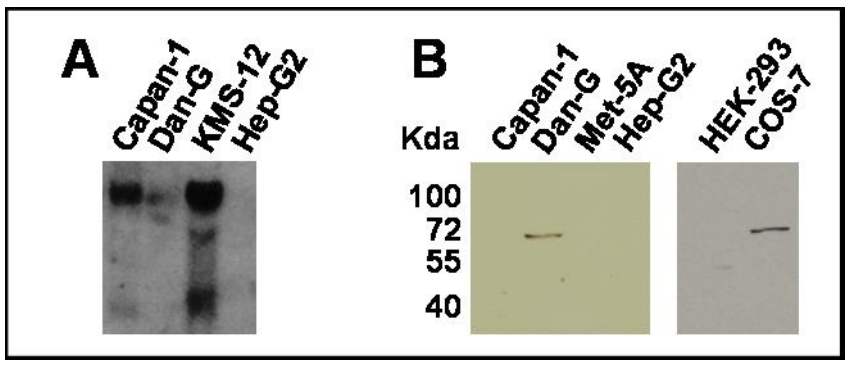



**Figure 3.26. Northern and Western blot analysis of adenocarcinoma cell lines**
(A) Northern blot analysis of RNA isolated from the indicated cells. The RNAs were submitted to a formaldehyde gel and blotted with a $^{32}$P-labelled *myeov* fragment. (B) Western blot analysis of proteins extracted from different cell lines with MYEOV specific polyclonal antibody.

# 4. Discussion

## 4.1. Identification of the *myeov* gene

The *myeov* gene was identified in a tumorigenicity assay with DNA from a patient suffering from a gastric carcinoma. FISH analysis mapped the *myeov* gene to chromosome 11q13 (Janssen et al., 1999; Janssen et al., 2000). The *myeov* gene was also shown to be involved in cases of multiple myeloma (MM) harboring the t(11;14)(q13;q32). Further, FISH analysis revealed that *myeov* is activated in these MM patients through the juxtaposition of the Eμ enhancer of the immunoglobulin heavy chain to the *myeov* gene (Janssen et al., 2000). *Myeov* expression is tightly regulated. At first, it has been shown in cell lines of patients with esophageal squamous cell carcinomas that *myeov* transcription is repressed by gene methylation (Janssen et al., 2002). Secondly, our study demonstrated that MYEOV biosynthesis is also controlled by the presence of upstream AUG codons (uAUGs) present in the *myeov* 5`UTR.

Similar mechanisms have also been described for other important genes in which potent protein overproduction is repressed by the presence of uAUGs in the transcribed mRNAs. Along this line it may be suggested that the function of the MYEOV protein might be very important.

## 4.2. Protein-protein interaction

In order to clarify the function of the MYEOV protein and its possible role in carcinogenesis, we looked for proteins that may interact with the MYEOV protein. Unfortunately we failed to find any interaction partner in the yeast as well in the bacterial two hybrid system. Therefore, we cloned the *myeov* coding region in frame with GST and myc tags in order to perform coimmunoprecipitation analysis and to detect and isolate possible interaction partners biochemically. These experiments were also negative and did not allow us to find any interaction partner of the MYEOV protein.

## 4.3. MYEOV does not code for a transcription factor

As the MYEOV protein does not contain any known motifs or domains and we were unable to detect any interaction partner, its biological function remains enigmatic. A sequence homologous to the *myeov* cDNA sequence reported similarity to the transcription factor forkhead box D2. In order to test whether the MYEOV protein might function as a transcription factor, we used a two-hybrid like system. The *myeov* coding region failed to demonstrate any activation of the Firefly luciferase reporter gene in this assay. The very strong activity of Firefly luciferase measured in the lysate from cells transfected with control DNA confirmed that the system worked properly.

Data base analysis as well DNA analysis by Southern Blot (Zoo blot) of different species revealed the presence of the *myeov* gene in humans and monkeys only. The absence of the *myeov* gene in cat, rat, mouse, sheep and mice suggests that the *myeov* gene may play an important role in higher mammals.

## 4.4. Characterization of the *myeov* 5`UTR

The 5`UTR of most eukaryotic mRNAs has a length in the range between 100 and 300 nucleotides, which is compatible with the ribosome-scanning model (Kozak, 1999). In contrast, about 10% of the eukaryotic mRNAs have a longer atypical 5`UTR which may also contain upstream AUGs codons (uAUGs) and, in some cases, associated upstream open reading frames (uORFs) (Morris and Geballe, 2000). Interestingly, uAUGs are extremely common in certain class of genes, including two-thirds of oncogenes and other genes involved in the control of cellular growth and differentiation (Kozak, 1987a; Kozak, 1991a; Morris, 1995). These mRNAs are poorly translated by a cap-dependent translation mechanism (Willis, 1999). Translation initiation of almost all eukaryotic mRNAs proceeds by a cap-dependent mechanism whereby the AUG nearest the 5`UTR-end is utilized as initiation codon (Kozak, 2000; Pestova et al., 2001; Sedman et al., 1990). The control of mRNA translation is an important step in order to regulate gene expression, and most

of the translational regulation occurs at the level of initiation, which is usually the rate-limiting step in protein synthesis (Donahue, 2000; Hershey, 1991; Kozak, 1991c; Mathews et al., 2000; Sonenberg, 1994).

The *myeov* 5`UTR has several features which seem to be incompatible with an efficient ribosome scanning of protein synthesis initiation. 1) The *myeov* 5`UTR contains an unusual length of 445 nucleotides, 2) its 5`UTR has four upstream start codons (uAUGs), associated with four upstream open reading frames (uORFs) of 22, 59, 11 and 7 amino acids, respectively and 3) it has a high G+C content and can potentially form complex secondary structures. These features are common among mRNAs regulated by their 5`UTR and suggest that MYEOV protein synthesis might be regulated at a posttranscriptional level. These findings prompted us to investigate the possible role of the *myeov* 5`UTR in controlling its protein level.

Using the mfold 3.1 algorithm (Zuker, 2003) the *myeov* 5`UTR sequence was predicted to be able to form a very stable secondary structure with a Gibbs free energy of $\Delta G$ of -153,1 kcal/mol for the most stable configuration. It has been described that secondary structures caused by the high G+C content of vertebrate leader sequences attenuate ribosome scanning (Kozak, 1991a; Kozak, 1992). However, G+C rich elements can also serve as internal promoters for transcription (Kozak, 1991a; Macleod et al., 1998). In this situation the effect depends on the position of the hairpin structure to the AUG start codon. It was reported that an AUG codon in a suboptimal context was recognized better when a hairpin structure was positioned 13 to 15 nucleotides downstream of the AUG start codon (Kozak, 1990). This data fits with mapping experiments in which the leading edge of the ribosome was shown to extent about 15 nucleotides beyond the AUG codon (Kozak, 1977). A hairpin positioned at +15 would therefore be expected to pause the 40S ribosomal subunit with its AUG-recognition-center right over the AUG codon (Kozak, 1990). As the *myeov* 5`UTR structure is incompatible with the ribosome-scanning model, it is suggested that the *myeov* 5`UTR RNA secondary structure may block ribosome scanning, and consequently reduce or completely abrogate MYEOV protein translation. In such a case, MYEOV

protein synthesis would be regulated at a translational level, and/or might occur by a cap-independent mechanism (e.g. internal ribosome entry).

## 4.5. *Myeov* does not cotain an IRES

Some viral RNAs use an internal ribosome entry site (IRES) to initiate translation in a cap-independent manner. The RNA of these viruses is usually uncapped therefore utilizing an IRES sequence to recruit the ribosome to the vicinity of the initiation codon and facilitate translation. IRESes were also identified in mammalian mRNAs containing a cap-structure, e.g. *BiP*, the gene that codes for the immunoglobulin heavy chain binding protein, fibroblast growth factor 2 (*FGF2*), the X-chromosome linked inhibitor of apoptosis (*XIAP*), and *c-myc (Holcik and Korneluk, 2000; Macejak and Sarnow, 1991; Nanbru et al., 1997; Vagner et al., 1995; Yang and Sarnow, 1997)*. The IRESes found in these genes are located in their 5`UTRs near the start codon of the main open reading frame. In these examples, the ribosome circumvents the stable secondary structure or the uAUGs that may block ribosome-scanning by jumping on the RNA at the IRES directly or near to the AUG start codon, and initiate protein synthesis.

Transfection of DNA constructs containing the full *myeov* cDNA did not yield sufficient MYEOV protein to be detected by specific antibodies in Western blot analyses. When we inserted the *myeov* 5`UTR cDNA sequence into a monocistronic reporter construct (pGL3) upstream of the Firefly luciferase cistron, a strong reduction of the reporter gene activity was observed, suggesting that the stable secondary structure of its 5`UTR may have an attenuating effect on protein synthesis. Presence of a stable hairpin upstream the Firefly luciferase cistron in the monocistronic constructs (phpL) abrogated the translation of the reporter gene. In contrast, insertion of the *myeov* 5`UTR 3`of a stable hairpin stimulated the reporter activity 38 to 48 fold (Figure 3.6), suggesting that the secondary structure present in the *myeov* 5`UTR initiates MYEOV translation by internal ribosome entry or represents an internal promoter. To investigate if *myeov* contains IRES elements, the 5`UTR

was first inserted into the intercistronic region of the bicistronic vector pRF. The insertion of the *myeov* 5`UTR in the bicistronic construct also stimulated Firefly luciferase activity.

However, recent publications have shown that results from the bicistronic assay alone are not enough to prove that a sequence under scrutiny encompasses an IRES. Another possibility, for example, could be ribosomal readthrough enhanced by signals in the *myeov* 5`UTR. To examine the possibility of ribosomal readthrough from the first cistron, a stable hairpin was inserted upstream of the 3`cistron (Renilla luciferase) into the bicistronic vector pRF, creating phpRF. The presence of a hairpin abrogated luciferases activity. However, insertion of the *myeov* 5`UTR upstream of Firefly luciferase in the vector phpRF promoted strong induction of Firefly luciferase activity. These data suggested the presence of an IRES in the *myeov* 5`UTR. However, still other possibilities could explain the increased activity of the Firefly luciferase (Figure 4.1), like for example:

A) The possibility of RNA nuclease cleavage within the intercistronic region, creating two monocistronic mRNAs rather than the expected bicistronic mRNA (Figure 4.1a) (Jackson et al., 1995; Kozak, 2001b).

B) Another potent artifact reported using the bicistronic vector is the possibility that the putative IRES element contains a cryptic promoter which could result in the production of two capped monocistronic mRNAs (Figure 4.1b) (Jackson et al., 1995; Kozak, 2001b).

C) A third possibility is the presence of a splice acceptor site in the putative IRES sequence, resulting in this way in a form of mRNA that could be translated by a normal cap-dependent mechanism, and/or leaky scanning and/or ribosome reinitiation (Figure 4.1c) (Kozak, 2002b).

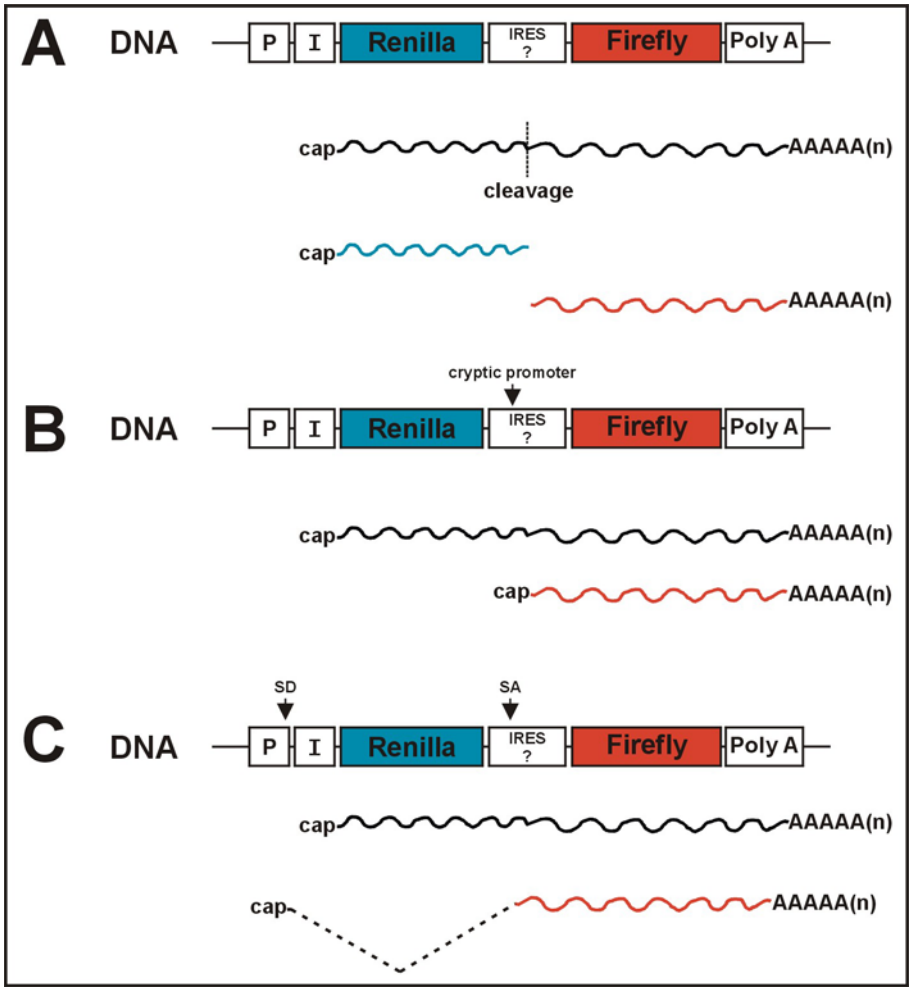



**Figure 4.1. Alternative mechanisms that may also increase the activity of the second cistron using the bicistronic assay to test sequences for IRES activity (see text). SD = splice donor and SA = splice aceptor.**

To distinguish between these possibilities, we performed additional control experiments, namely 1) in vitro transcription and translation of the bicistronic construct harboring the *myeov* 5`UTR; 2) deletion of the promoter in the mono and bicistronic constructs, creating promoterless constructs; 3) Northern blot analysis of RNAs from cells transfected with these constructs.

The results from the *in vitro* assay did not demonstrate the presence of an IRES in the *myeov* 5`UTR, since no increase in Firefly luciferase activity was detectable when compared to the empty vector pRF. Although the failure to demonstrate IRES activity in the *myeov* 5`UTR by this method argues against the presence of an IRES, this result might be explained by the requirement of IRES trans-activating factors (ITAFs) absent in this *in vitro* system. Some other

cellular IRESes were also inactive in this *in vitro* assay, suggesting the requirement of ITAFs for some internal ribosomal entry events (Holcik et al., 2003; Holcik and Korneluk, 2000; Kullmann et al., 2002; Millard et al., 2000; Pilipenko et al., 2000). For example, poliovirus and rhinovirus IRESes are not active in the rabbit reticulocyte lysate unless the lysate is supplemented by a HeLa cell extracts (Borman et al., 1993; Brown and Ehrenfeld, 1979; Dorner et al., 1984). Another explanation is that some cellular IRESes need a "nuclear event" to work properly (Iizuka et al., 1995; Stoneley et al., 2000). This is the case for the IRES of the antiapoptotic *XIAP*, which needs two nuclear ribonucleoproteins for proper IRES function (Holcik et al., 2003).

## 4.6. Analysis of the putative *myeov* IRES activity during cellular stress situations

Cap independent mechanisms mediated by an IRES are usually activated under situations where cap dependent mechanisms are reduced or completely blocked. For example, cap-dependent protein translation in cells infected by poliovirus is strongly impaired, but the viral mRNAs are still translated by a cap-independent translation mechanism that is mediated by an IRES (Macejak and Sarnow, 1991; Sarnow, 1989; Yang and Sarnow, 1997). The same is true for mRNAs that are translated in specific situations, e.g. during hypoxia, apoptosis, heat shock, etc. Also, during mitosis cap-dependent translation is impaired, suggesting a cap independent mechanism of translation for mRNAs expressed during this stage of the cell cycle (Hernandez et al., 2004; Pyronnet et al., 2000; Qin and Sarnow, 2004; Subkhankulova et al., 2001). Translation of ornithine decarboxylase (ODC), the rate-limiting enzyme in the biosynthesis of polyamines, peaks twice during the cell cycle, at the G1/S transition and at G2/M (Pyronnet et al., 2000). It has been described that an IRES in the *ODC* mRNA that functions exclusively at G2/M is present to ensure elevated levels of polyamines, which are necessary for mitotic spindle formation and chromatin condensation (Pyronnet et al., 2000). The *c-myc*

mRNA also contains an IRES that functions specifically during mitosis (Subkhankulova et al., 2001).

To check whether the putative *myeov* IRES is activated during such stress situations, we investigated the effect of the *myeov* 5`UTR during cell starvation and apoptosis. Cells transfected with the DNAs from the bicistronic constructs containing the *myeov* 5`UTR were treated with Staurosporin, which is a strong inhibitor of protein kinase C and consequently has a potent toxic effect in cells, leading to apoptosis. Analysis of luciferase activities in these cells did not show increased Firefly luciferase activity when compared to untreated cells (Figure 3.11). This result was confirmed in cells transfected with the bicistronic constructs containing the *myeov* 5`UTR that were co-transfected with FADD, a death domain-containing protein that interacts with the death domain of Fas and initiates apoptosis. In the group of cells transfected with DNAs of the reporter gene, which were deprived of serum, we also did not see any induction of Firefly luciferase activity (Figure 3.12). Together these data suggested that either the *myeov* putative IRES is not active during cellular stress situations or the *myeov* 5`UTR does not contain an IRES.

## 4.7. *Myeov* 5`UTR harbours a cryptic promoter

We next investigated the possibility whether the *myeov* 5`UTR contains a cryptic promoter by removing the SV40 promoter of the mono- and bicistronic constructs. The activity of the Firefly luciferase in transfected cells with DNA of the promoterless constructs, suggested the presence of a cryptic promoter in the *myeov* 5`UTR. This result was confirmed by Northern blot analysis of RNAs from transfected cells, using a Firefly luciferase fragment as a probe. In these Northern Blot analyses we detected a transcript the size of which suggests a transcriptional start site within the *myeov* 5`UTR. This data was also confirmed using the bicistronic construct (pRF+UTR) that contains the *myeov* 5`UTR and the SV40 promoter. In this case, two transcripts were observed. The first transcript initiated at the SV40 promoter, and the second

one initiated within the intercistronic region, and therefore presumably in the *myeov* 5`UTR. Hybridization of the Northern Blot with a 5` Renilla luciferase probe showed only one transcript starting from the SV40 promoter. The absence of a second and smaller transcript confirmed our findings that the second transcript originated in the *myeov* 5`UTR. Surprisingly, these analyses also revealed the presence of promoter activity in the 5`UTR of EMCV that was confirmed by transfection of promoterless constructs. The 5`UTR of EMCV harbors an IRES (Jang et al., 1988), as published in numerous studies (Aminev et al., 2003; Davies and Kaufman, 1992; Dove et al., 2004; Kolupaeva et al., 2003; Urwin et al., 2000; Witherell and Wimmer, 1994). Even our DNA transfections experiments using the bicistronic reporter construct, as well as the *in vitro* transcription and translation experiments also suggested the presence of an IRES in the 5`UTR of EMCV.

There is an ongoing debate whether cellular mRNAs really contain IRESes. In a recent review Kozak discussed 26 publications of cellular mRNAs that were reported to contain an IRES (Kozak, 2001b). She criticized the design of different experiments performed to identify the putative IRES in those mRNAs. According to this report only one of the 26 reported putative IRESes may contain an IRES, while in all the other cases the mRNAs were probably translated by an alternative transcript derived from splicing that would remove part of their 5`UTR or the presence of a cryptic promoter in their 5`UTR. Our results are in agreement with her criticism and similar observations were also described by other investigators, e.g. for the translation of the eIF4G that originally was reported to be mediated by an IRES located in its 5`UTR (Gan et al., 1998; Johannes and Sarnow, 1998). Experiments using promoterless constructs ruled out the presence of an IRES in the 5`UTR of eIF4G, but rather revealed the presence of cryptic promoter activity (Han and Zhang, 2002). Although RNA analysis performed by Northern blot were usually conducted to rule out the existence of monocistronic transcript originating from the second cistron, monocistronic transcripts that represent less than 5% of the bicistronic mRNA level may not be detected by Northern blotting analysis (Kozak, 2001b). Therefore, inadequate RNA analysis may explain the inflation of

reports on cellular mRNAs containing putative IRESes. Consequently, some of the reported cellular IRESes might be erroneously identified. For example, the mRNAs of *ornithine decarboxylase* (Pyronnet et al., 2000), *connexin-32* (Hudder and Werner, 2000) and *Gtx* (Chappell et al., 2000) have been reported to contain an IRES, since their 5`UTR supported translation of the 3` cistron of the bicistronic reporter construct 2.5, 5 and 7 fold, respectively, when compared to the empty vector. This marginal induction of reporter gene activity might also be explained by "contaminated" monocistronic RNA that is not detectable by Northern blot analysis. In agreement with that, the human *Sno* and mouse *Bad* mRNAs were described to contain an IRES in their 5`UTR, and Northern blot analysis of their mRNA did not reveal the presence of a monocistronic transcript and thus supported this view. Unfortunately, the promoterless assay revealed the presence of a cryptic promoter in both 5`UTR`s (Han and Zhang, 2002). The different results obtained using promoterless constructs, support the view that it is of outmost importance to use this system when analyzing putative IRES sequences to rule out the presence of a cryptic promoter in the sequence under scrutiny. Our results impressively support this argument, since we detected promoter activity in the *myeov* 5`UTR using the promoterless constructs.

Deletions of the *myeov* 5` UTR suggested that the cryptic promoter drives transcription of a mRNA that still encompasses two or even three AUGs upstream of the *myeov* main ORF. Presently, we are mapping the precise transcription start site of this *myeov* cryptic promoter by primer extension analysis.

## 4.8. *Myeov* uAUGs reduce translation of the reporter gene

Despite the strong promoter activity detected in the *myeov* 5`UTR, the reporter gene activity was rather low due to the presence of four upstream AUGs (uAUGs) within the *myeov* 5`UTR. This was confirmed by mutation analysis. Transfection of wild type *myeov* 5`UTR, strongly reduced reporter gene activity. In contrast, in a mutated form, in which all four uAUGs were

removed, the protein level was almost restored to the level of the control construct pRF+EMCV (Figure 3.19). This result was also confirmed by directly transfecting RNAs into cells. The biggest advantage of this method is that it bypasses the complex issue of transcriptional regulation, and the effects are directly measured after the cytoplasmic delivery of the transcripts (Han and Zhang, 2002). These results suggested that the absence of upstream start codons allowed the ribosome to reach the reporter gene start codon. In order to investigate if this translational control can be assigned to a specific uAUG, we have created 16 mutational combinations. Specific analysis of the reporter gene activity in transfected cells revealed that all four *myeov* uAUGs had some effect on protein translation control, but a preferable uAUG could not be clearly observed.

Attenuation of translation efficiency by the presence of uORFs is well known for genes having a critical proliferation or survival function, including oncogenes, cytokines, signal transduction proteins, transcription factors, and other potent proteins (Hernandez-Sanchez et al., 2003; Morris and Geballe, 2000). In the case of cellular mRNAs, uORFs might be used as a device for limiting the translation of potent proteins that would be harmful if overproduced (Kozak, 2001a). When the protein of the major ORF would be required, the inhibitory effect of the uORF will be eliminated by switching to an alternative promoter or splice site (Arrick et al., 1994; Phelps et al., 1998).

The most well described example concerning uORFs is the yeast *GCN4* gene. This gene encodes a transcription factor that is responsible for the activation of the expression of approximately 50 genes of the amino acid biosynthesis (Grant et al., 1995). During amino acid deprivation, protein synthesis is blocked and in turn translation of the GCN4 mRNA is enhanced (Hinnebusch, 1996). The *GCN4* mRNA contains four uORFs that are necessary for *GCN4* protein translation control in response to amino acid limitation (Hinnebusch, 1996; Hinnebusch, 1997). The uORF1 is always translated efficiently, after which ribosomes resume scanning and reinitiate, usually at uORF4. uORF4 is unusual in that its translation precludes further reinitiation events, thus, when uORF4 is translated, GCN4 is not. This is the situation in

which yeast cultures contain adequate nutrients. In contrast, amino acid starvation causes some ribosomes to bypass the inhibitory uORF4 and to reinitiate instead farther downstream. This occurs because starvation creates a pool of uncharged tRNAs which activate a protein kinase that phosphorylates, and thus partially inactivates, eIF2. When eIF2 levels fall, it takes longer for ribosomes to reacquire Met-tRNA$_i$ and thus become competent to reinitiate. The slower acquisition of competence means that some ribosomes, scanning in the reinitiation mode, will bypass the nearby uORF4 and can thus reach the downstream *GCN4* start site (Hinnebusch, 1996; Hinnebusch, 1997; Kozak, 2002b).

A second example is the proinsulin that is expressed prior to development of the pancreas and promotes cell survival. The embryonic proinsulin mRNA shares the coding region with the pancreatic form, but presents a 32 nucleotides extension in its 5`UTR. This extension contains two uAUGs which are responsible for the reduced level of insulin protein. The presence of these uAUGs is necessary to ensure a low insulin level, since the addition of exogenous proinsulin to embryos in *ovo* decreases apoptosis and in turn generates abnormal developmental traits. This data shows the significance of uAUGs as a tight regulator of protein levels important for developmental signal pathways (Hernandez-Sanchez et al., 2003).

There are several other examples where uAUGs are responsible for the inhibition of protein translation. Thrombopoietin protein production is strongly impaired by the presence of uAUGs, resulting in very low thrombopoietin a level that is normally found in serum. Mutation or splicing events that remove the uAUGs can lead to overproduction of this potent cytokine, resulting in thrombocythaemia (Ghilardi et al., 1998). This was confirmed in a study performed in a Dutch family. This family comprised eleven individuals with thrombocythaemia (Wiestner et al., 1998). The thrombopoietin (TPO) long transcript contains seven uAUG codons upstream the TPO ORF. The uAUG1 through uAUG6 create small upstream open reading frames which terminate before the main TPO start codon, while the uAUG7 produces a small open reading frame that overlaps with the TPO start codon. All uAUGs contribute to

translation control of TPO protein synthesis. The thrombopoietin gene of the affected individuals in the described Dutch family contains a mutation which leads to shortened 5` UTR removing specific uAUG codons, which leads to overproduction of the thrombopoietin protein. The absence of the uAUG codon was considered the responsible factor to cause this thrombocythaemia (Kozak, 2002a; Wiestner et al., 1998). In conclusion, hereditary thrombocythaemia result from mutations that restructure the 5` leader sequence in a manner that promotes elevated level of this potent cytokine (Ghilardi et al., 1998; Kondo et al., 1998; Wiestner et al., 1998). Additional examples of the involvement of uORFs in translation control in human diseases are described in the review of Kozak 2002 (Kozak, 2002a).

## 4.9. *Myeov* uAUGs control MYEOV biosynthesis

Our data clearly showed that the uAUGs located in the *myeov* 5`UTR strongly impaired the translation of the reporter gene. Next, we wanted to find out the effect of the uAUGs on the translation of the *myeov* ORF. Analysis of RNAs and proteins from transfected cells using the complete *myeov* cDNA wild type or the mutated form, where all four uAUGs were mutated, confirmed our previous results where we used the mono- and bicistronic reporter constructs. It has also been demonstrated that the 3`UTR can also participate in controlling protein translation initiation (Gallie, 1996; Gebauer et al., 1999; Jacobson, 1996; Michel et al., 2000; Valcarcel and Gebauer, 1997). This is in part due to the interaction between the Poly(A) tail with the PAB protein, which again interacts with the initiation factor eIF4G. The cap-binding initiation factor, eIF4E on its turn interacts with the factor eIF4G. In this way, both mRNA ends bind to the eIF4G through interaction of the eIF4E and PAB protein. Contacts between the eIF4G and the poly A tail bound to PAB seem to enhance translation efficiency but are not absolutely required for ribosome recruitment (von der Haar et al., 2004). To clarify whether the *myeov* 3`UTR collaborates in MYEOV protein translation control, a myeov cDNA construct in the eukaryotic expression vector pMT2SM lacking its 3`UTR was used. Absence

of the *myeov* 3`UTR did not alter the protein levels as no protein could be detected by Western Immunoblotting analysis. This was confirmed with the construct lacking the *myeov* 5`UTR, but still containing the *myeov* ORF and its 3`UTR. In cells transfected with DNA from this construct, MYEOV protein could be detected by Western Immunoblotting using MYEOV specific antibodies.

Our results clearly demonstrated that MYEOV protein translation is strongly impaired by the presence of uAUGs or by the small uORFs. In situations where efficient translation of MYEOV protein is required, the inhibitory effect of uAUGs or uORFs may be eliminated by switching to an alternative promoter or splice site (Phelps et al., 1998). There are several examples in which a specific gene creates transcripts with different 5`UTRs. In mice, the cyclin-dependent kinase inhibitor $p18^{INK4c}$ is excessively transcribed but not translated because the mRNA contains a long 5`UTR with 1115 nucleotides and five uAUGs. During differentiation, a downstream promoter produces a second transcript with a smaller mRNA that can be translated efficiently (Phelps et al., 1998). The human *MET* gene ORF encodes a tryrosine kinase receptor that binds the human growth factor (HGF) and is implicated in oncogenesis. The mRNA of the *MET* gene is present in all cell types, but it has been demonstrated to lack the exon that contains the start codon (Lin et al., 1998). The inability to translate this transcript is a way to adjust the quantity of functional mRNA for this important protein (Lin et al., 1998). The mouse cerebroside sulfotransferase gene is transcribed in many tissues and is also not translated because of the presence of uAUGs (Hirahara et al., 2000). These examples show that it is important to verify whether alternative transcripts removing uAUGs from a mRNA exist. However, detection of such alternative transcripts is complicated by 1) competition for a common probe that can cause minor transcripts to be missed (Chen et al., 1999), 2) even abundant transcripts can be missed just because the probe was located too far upstream (Cortner and Farnham, 1990) or within an intron (Frost et al., 2000) or 3) because hybridization conditions favored detection of a minor GC-rich 5`UTR over an alternative AU-rich 5`UTR (Sazer and Schimke, 1986).

## 4.10. Expression of MYEOV protein in carcinoma cell lines

We have also analyzed several carcinoma cell lines by Western blot analysis using MYEOV specific antibodies. We found that some pancreas carcinoma cells expressed MYEOV protein, albeit of an abnormal size. This difference may be explained by the presence of anomalous transcripts that are produced in pancreas tissue. In fact, in a previous study we reported the presence of alternative *myeov* transcripts in pancreas tissue. These pancreas specific mRNAs may differ in their 5`UTR sequences and regulate MYEOV biosynthesis in a tissue specific manner. The regulation of MYEOV biosynthesis and its biological function, as well as the presence of specific MYEOV protein products in human pancreas deserves further attention and hopefully will be clarified in future studies.

# 5. Summary

The *myeov* gene was identified using the tumorigenicity assay with DNA from a patient suffering a gastric carcinoma. The *Myeov* gene is localized at chromosome band 11q13, a frequent site for chromosomal rearrangements in various carcinomas and B-cell neoplasms. The gene was shown to be involved in cases of multiple myeloma harboring the t(11;14)(q13;q32). In addition, *myeov* is coamplified with *cyclin D1* and overexpressed in carcinomas of the breast, lung, bladder, esophageal squamous cell carcinomas and oral squamous cell carcinomas. *Myeov* DNA amplification and overexpression was detected in several carcinoma cell lines, however, hardly any MYEOV protein could be detected using specific antibodies in Western blot analysis. The 5` untranslated region (5`UTR) of the *myeov* gene is long, encompasses four upstream AUG codons (uAUGs) and is predicted to fold in a strong secondary structure. These features are common among mRNAs regulated by their 5`UTR and suggest that MYEOV protein synthesis might be regulated at a posttranscriptional level. These findings prompted us to investigate the possible role of the *myeov* 5`UTR in controlling its protein level, and the possibility of MYEOV protein synthesis to be mediated by an internal ribosome entry site (IRES). Here we show that initial experiments using mono- and bicistronic reporter constructs supported this view. However, further examination by *in vitro* transcription/translation assays, Northern blot analysis and the application of promoterless constructs revealed promoter activity in the myeov 5`UTR. Despite this strong promoter activity, we did not find any translation products. Our experiments showed that this was due to the presence of uAUGs codons present in the myeov 5`UTR. DNA and RNA transfection of the wild type and mutated 5`UTR, where the uAUGs were mutated to AAG, confirmed that these uAUGs abrogate translation of the reporter gene as well as the *myeov* gene. Alternative splicing mechanisms in specific cell types and/or developmental stages may be a way to evade this translation control.

# 6. Zusammenfassung

Das *Myeov* Gen wurde mittels eines Tumorigenizitätsassays aus der DNA eines Patienten mit einem Magentumor isoliert. Das Gen ist auf der Chromosomenbande 11q13 lokalisiert worden, einem häufig betroffenen Abschnitt für chromosomale Rekombinationen in verschiedenen Karzinomen und B-Zell-Neoplasien. Es ist nachweislich an Fällen multipler Myelome beteiligt, die eine t(11;14)(q13;q32) zeigen. Auch findet man *Myeov* zusammen mit *Cyclin D1* koamplifiziert und überexprimiert in Karzinomen der Brust, der Lunge, der Blase, sowie in Epithelzellkarzinomen des Ösophagus und der Mundhöhle. Amplifikation und Überexpression von MYEOV wurden zwar in mehreren Karzinom-Zelllinien gezeigt, jedoch konnte in der Western-Blot-Analyse mit myeov-spezifischen Antikörpern kein Protein nachgewiesen werden. Die 5` nichttranslatierte Region (5`UTR) des *Myeov*-Gens ist relativ lang, sie enthält vier vorgeschaltete AUG Kodons (uAUGs) und ist laut Prognose eines Computerprogramms in der Lage, eine starke Sekundärstruktur zu bilden. Diese Eigenschaften kommen häufig bei mRNAs vor, die durch ihre 5`UTR reguliert werden und deuteten auch in unserem Fall darauf hin, daß die MYEOV-Proteinsynthese auf postranskriptionaler Ebene gesteuert sein könnte. Diese Entdeckungen veranlassten uns dazu, einerseits die mögliche Rolle der *Myeov*-5`UTR bei der Regulation der Proteinexpression näher zu untersuchen, sowie andererseits zunächst die Möglichkeit zu überprüfen, ob die Proteinsynthese durch eine „internal ribosome entry site“ (IRES) ermöglicht werden könnte. Erste Experimente mit mono- und bicistronischen Reporterkonstrukten schienen diese Ansicht zu stützen. Jedoch wies die weitere Prüfung durch in vitro-transcription/translation assays, Northern-Blot-Analyse und die Verwendung promotorloser Konstrukte auf eine Promotorfunktion der *Myeov* 5`UTR hin. Doch trotz deutlicher Promotoraktivität fanden wir keine translatierten Produkte. Unsere Experimente zeigten schließlich, daß die Ursache in den uAUG Kodons der *Myeov* 5`UTR zu suchen war. Die Transfektion verschiedener DNA- und RNA-Luciferase- Reporterkonstrukte mit *Myeov* 5`UTR und parallel dazu einer mutierten Form, bei der die uAUGs zu AAG verändert worden waren, bestätigte, daß diese uAUGs die Translation des Reportergens sowie des *Myeov*-Gens verhindern. Alternatives Splicing in bestimmten Zell-Arten und/oder Entwicklungsstadien könnten eine Möglichkeit sein, diese Translationskontrolle zu umgehen.

# 7. Acknowledgments

First of all, I would like to thank Prof. Dr. C.R. Bartram for giving me the opportunity to work for my doctoral thesis at the Institute of Human Genetics in the Medical Faculty of the Ruprecht-Karls University of Heidelberg.

I am extremely grateful to PD Dr. J.W.G. Janssen for patiently supervising me during these years, especially during the preparation of this thesis and for his speed and valuable suggestions throughout the project.

I would like to thank Prof. Dr. W. Buselmaier for acting as the advicer and the first referee of my Ph. D. work. I also thank Prof. Dr. H. Steinbeisser for critically reviewing my thesis and for being the second referee of this work.

I would like to express my gratitude to Dr. R. Blaschke for editing my thesis and for his helpful tips during my work.

I appreciate all my colleagues in the lab: Dr. Martina Schrank, Christian Schaaf, Thomas Schmitt and Susanne Luf and especially Dorothee Erz who kindly shared her experience with me and for her expert technical assistance. I am thankful to Marcus Brecht for the good time we had working together in the lab and for his dedication for trying to help me improving my German language. I am grateful to Jianli Guo for her friendship and for sharing her knownledge in biochemistry.

I thank Dr. Tutaka Shimada (Department of Surgery, Kyoto University Hospital, Japan) for providing important patients' material. I also thank Dr. Anne Willis (Department of Biochemistry, University of Leicester, England) for providing the different luciferase vectors that were very important for my work.

I would like to thank Dr. Ying Yang, Nitin Sabherwal and Mrs. Anne Jordan. I am thankful to Pawel Licznerski for being a friend and for sharing his great expertise.

Especially I want to thank my good friend Elizabeth Stockmann who gave me the idea to study in Germany and for her encouragement during all these years.

And last but not the least, I am grateful to my family and all friends whose names are not mentioned here, for their affection, love and support during all this duration.

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