Regulation of Translation Initiation at the Poliovirus IRES

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Summary

Poliovirus (PV) translation and replication can occur in neuronal cells where it causes degeneration and lysis of cells leading to paralytic poliomyelitis. Other cell types are much less affected by PV infection and do not support translation and replication of the virus as well. Apart from the poliospecific receptor, the reasons for the tissue preference of poliovirus may be found in its translation initiation via an internal ribosome entry site (IRES), which in addition to cellular initiation factors uses proteins normally not involved in translation to achieve efficient translation (ITAFs). Several ITAFs are known, but neither do these known factors explain the tissue specificity of poliovirus nor are they sufficient to initiate translation at the PV IRES using only purified components. Additional factors may be present in cells, especially in neuronal cells, that are both necessary for translation initiation on the PV IRES.

One potential new factor, ErB3 binding protein (EbP1), was identified here to bind to the PV IRES. Translation initiation complexes were formed with labelled Poliovirus IRES RNA in an in vitro reconstitution system. A double labeled RNA allowed to identify 48S complexes in sucrose gradients and to enrich proteins present in these complexes. As controls RNAs containing the IRESs of Hepatitis C virus (HCV) and encephalomyocarditis virus (EMCV) were used. One protein, specific for PV and EMCV was identified in the light RNA peak but not in the 48S peak. It was identified by mass spectrometry as EbP1, which is a known ITAF for the IRES of Foot-and-mouth-disease virus. However, its role in PV translation initiation seems to be inhibiting rather than stimulating since it could not be found in 48S complexes.

Additionally I identified a neuron specific microRNA (miRNA) that stimulates translation at the Poliovirus IRES. By screening the poliovirus IRES sequence for potential target sites for miRNAs that are preferentially expressed in neuronal cells, I found sequences partially complementary to three neuron-specific miRNAs, miRNA-127, miRNA-326 and miRNA-422a. These miRNAs were co-transfected into cultured cell-lines together with a poliovirus IRES reporter RNA. Of these miRNAs, only miRNA-326 enhanced poliovirus translation efficiency. The target site for miRNA-326 is located in a loop of the central IRES domain IV. Mutagenesis of this miRNA-326 target site disabled the stimulation by added miRNA-326, indicating a physical interaction of miRNA-326 with the poliovirus IRES. However, this mutation could only be partially rescued by a miRNA with a complementary mutation in the seed sequence of miRNA-326. Since two binding sites of miRNA-326 in the 5' untranslated region of PV are identical with binding sites for the known ITAF PCBP2, a connection between those two factors may be assumed.

Zusammenfassung

Poliovirus (PV) Translation und Replikation findet in neuronalen Zellen statt. Infizierte Zellen werden degradiert oder lysiert, was zum Krankheitsbild der Kinderlähmung führt. Andere Gewebe sind von Polioinfektionen weit weniger betroffen, da sie Translation und Replikation des Virus nicht unterstützen. Der Grund für die Gewebespezifität von Poliovirus liegt neben dem Rezeptor auch an der Initiation der Translation, die mit Hilfe einer internen Ribosomen Eintrittsstelle (IRES) realisiert wird. IRES abhängige Translation benötigt neben zellulären Initiationsfaktoren auch andere zelluläre Proteine (ITAFs), welche normalerweise nicht an der Translation beteiligt sind. Mehrere ITAFs für PV sind bekannt, allerdings kann durch sie weder die Gewebespezifität von PV erklärt werden, noch sind all diese Faktoren ausreichend, um in aufgereinigter Form Translation durch die PV IRES zu initiieren. Es muss also noch weitere Faktoren geben, die für die Translation durch die PV IRES in neuronalen Zellen notwendig sind.

Ein neuer potentieller ITAF, ErB3-binding protein (EbP1), wurde in dieser Arbeit identifiziert. Dafür wurden Translationsinitiationskomplexe an der PV IRES gebildet. Eine doppelte Markierung erlaubt sowohl die Identifikation der 48S Komplexe im Gradienten, als auch eine Isolierung von RNA bindenden Proteinen aus diesen Komplexen. Als Kontrolle wurden RNAs mit der Hepatitis C Virus und der Encephalomyocarditis Virus (EMCV) IRES eingesetzt. Ein Protein konnte in den leichten Sucrose Fraktionen an der PV und EMCV IRES beobachtet werden, jedoch nicht in den 48S Komplexen. Dieses Protein wurde durch Massenspektrometrie als EbP1 identifiziert. EbP1 wurde bereits als ITAF für das Maul- und Klauenseuche Virus identifiziert.

Zusätzlich wurde eine neuronen-spezifische mikroRNA (miRNA) identifiziert, welche die Translation an der PV IRES stimuliert. Dafür wurden mehrere neuronen-spezifische miRNAs, miRNA-127, miRNA-326 und miRNA-422a ausgewählt, die Bindestellen in der PV IRES haben. Diese miRNAs wurden zusammen mit einem PV IRES Reporterkonstrukt in Zelllinien transfiziert. Nur miRNA-326 zeigte eine Stimulation der Translation. Die Bindestelle für miRNA-326 befindet sich in einer nicht gepaarten Region innerhalb von Domäne IV in der Mitte der PV IRES. Mutagenese dieser miRNA-326 Bindestelle verhinderte die Stimulation durch miRNA-326. Der Effekt konnte jedoch mit einer kompensatorisch mutierten miRNA nur teilweise wieder hergestellt werden. Beide Bindestellen für miRNA-326 in der PV IRES sind mit Bindestellen für den ITAF PCBP2 identisch, daher kann ein Zusammenspiel dieser beiden Faktoren in der Translation angenommen werden.

Table of content

Abbreviations

ago	Protein of the argonaute family
ANOVA	Analysis of variables
Amp	Ampicillin
APS	Ammoniumpersulfate
ATP	Adenosin triphosphate
ARS	Autoregulatory sequence
bp	Base pairs
CBV3	Coxsackie B virus 3
CO_2	Carbon dioxide
cpm	Counts per minute
CrPV	Cricket paralysis virus
CTP	Cytosine triphosphate
CSFV	Classical swine fever virus
ddH_2O	Double distilled water
DEAE	Diethylaminoethane
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagle's medium
dNTP	Desoxyribonucleotide
ds	Double stranded
DTT	Dithiothreitol
ebP1	ErB3 binding protein
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
eIF4E-BP	eIF4E binding proteins
EMCV	Encephalomyocarditis virus
ErB3	Epidermal receptor tyrosine kinase
FBS	Fetal bovine serum
FMDV	Foot-and-mouth-disease virus
FPLC	Fast protein liquid chromatography
GTP	Guanosine triphosphate
HAV	Hepatitis A virus
HCV	Hepatitis C Virus

hnRNP	Heterogenous ribonucleoprotein
HRV	Human rhinovirus
IGR	Intragenic region
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITAF	IRES trans-acting factor
IRES	Internal Ribosome Entry Site
Kan	Kanamycine
KH	hnRNP K homology
LB	Luria-Bertani
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
miRNA	MicroRNA
mnk	Mitogen activated kinase
mRNA	Messenger RNA
mut	Mutation
NS	Non-structural
nt	Nucleotide
OD	Optical density
ORF	Open reading frame
PA	Polyacrylamide
PABP	PolyA-binding protein
PAGE	Polyacrylamide gel ectrophoresis
PAIP	PABP interacting protein
PBS	Phosphate buffered saline
PC	Phosphocellulose
PCBP	Poly r(C) binding protein
PCR	Polymerase chain reaction
PTB	Polypyrimidine tract binding protein
PV	Poliovirus
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rNTP	Ribonucleotid
rpm	Rounds per minute
RRL	Rabbit Reticulocyte Lysate

RRM	RNA recognition motif
RSW	Ribosomal salt wash
RT-PCR	Real-time PCR
SDS	Sodium dodecylsulfate
snRNP	Small nuclear ribonucleoprotein particles
SMN	Survival of motoneurons (complex)
sRNA	Small RNA
Ss	Single stranded
TBE	Tris, Borate, EDTA
TEMED	Tetramethylethylendiamine
TMV	Tobacco mosaic virus
TMEV	Theiler's murine encephalitis virus
u	Unit
UTP	Uridine triphosphate
UTR	Untranslated region
Vp	Viral protein
Wt	Wild type
XIAP	X-linked inhibitor of apoptosis

Prefixes	
Kilo (k)	x 10^{3}
Centi (c)	x 10^{-2}
Milli (m)	x 10^{-3}
Micro (µ)	x 10^{-6}
Nano (n)	x 10^{-9}
Pico (p)	x 10^{-12}

Units	
h	hour
min	minute
s	second
m	meter
l	liter

g	Gram
Da	Dalton
S	Svedberg (sedimentation coefficient)
V	Volt
A	Ampere
℃	Degrees Celsius

Introduction

Poliovirus

Poliovirus belongs to the group of picornaviruses; positive strand RNA viruses that include several human and animal pathogens, e.g. human rhinovirus (HRV), Hepatitis A virus (HAV), Encephalomyocarditis virus (EMCV) and foot-and-mouth disease virus (FMDV).

Poliovirus (PV) causes poliomyelitis, a severe motor neuronal disease, resulting in paralysis. The virus is transmitted fecal-orally and primarily infects gut cells. In less than 1 % of cases the infection spreads to the nervous system (Racaniello 2006), where it causes severe and permanent damage to motor neurons (Colbere-Garapin et al. 1989, Wood & Macadam 1997). Poliovirus (PV) infects only humans (LaMonica et al. 1986), even though some old world primates can be infected experimentally (Racaniello 2006).

The Poliovirus RNA genome (figure 1) contains a long 5' untranslated region (UTR or NTR) of approximately 750 nucleotides (nts), 450 of which form an internal ribosome entry site (IRES), followed by a single open reading frame (ORF) coding for the viral polyprotein which cleaves itself into 11 structural and non-structural proteins (Shih et al. 1978). The coding region is followed by a short 3' UTR of 65 nts (Waggoner & Sarnow 1998) and a polyA tail of 84 As (Semler et al. 1984). A more detailed explanation of structure and function of the internal ribosome entry site (IRES) will be given later in the text and in figure 3.

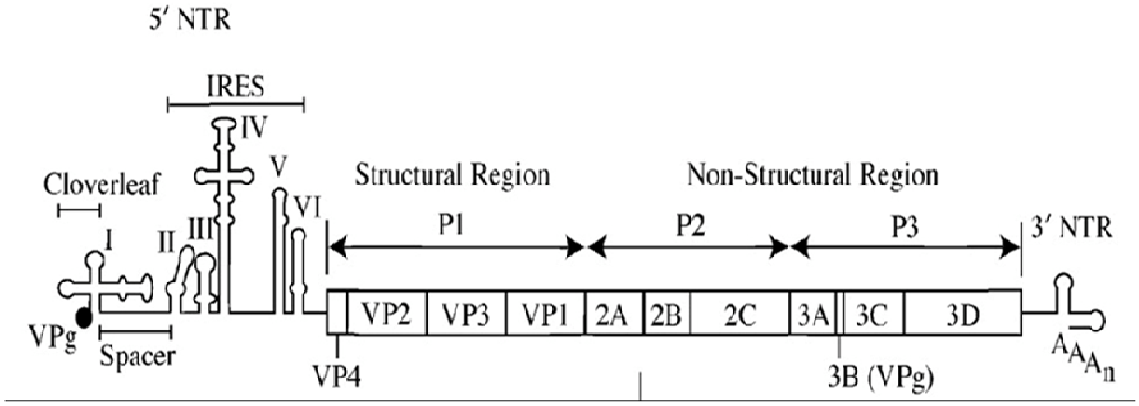

Figure 1: Organization of Poliovirus (PV) genomic RNA, NTR: non-translated region, VP: viral protein. Picture from DeJesus et al. 2007

Translation initiation

Protein biosynthesis is a universal process, necessary for all organisms on earth. RNA gets translated into proteins almost identically in eukaryotes, archea and bacteria (Kyrpides et al. 1998, Koonin et al. 2001, Laursen et al. 2005). Translation is divided into four parts: initiation, elongation, termination and ribosome recycling (Pacheco & Martinez-Salas 2010). Initiation is the rate-limiting step and therefore the most regulated one. It involves many factors and differs widely between the kingdoms of life (Kyprides et al. 1998).

Eukaryotic cap-dependent translation initiation

Most cellular mRNAs have a 5' cap structure, a methylated guanosine at the very 5' end of the mRNA (Shatkin et al. 1976, Sonenberg et al. 1979). The cap is added during transcription in the nucleus and serves a double function. It protects the mRNA from degradation and allows ribosomes to bind to the mRNA.

The cap is recognized by the eukaryotic initiation factor (eIF) 4F, which consists of 3 subunits, eIF4A, 4E and 4G (figure 2). Simultaneously the eukaryotic initiation factor eIF2 together with the starter methionyl- t-RNA and GTP form a ternary complex. The ternary complex then joins with eIF3 (Asano et al. 2000), eIF1, eIF1A and the 40S ribosomal subunit to form the 43S pre-initiation complex (Hinnebusch 2006). Binding of the 43S pre-initiation complex to the capped RNA via interaction of eIF4G and eIF3 leads to the 48S pre-initiation complex. eIF4G is connected to the mRNA via eIF4E, the cap-binding moiety of eIF4F, therefore connecting the 40S ribosomal subunit to the mRNA via eIF3.

The complex then scans along the mRNA towards the 3' end until it reaches the start codon, AUG, in a favorable Kozak-environment (Kozak 1989) normally 50-100 nts downstream of the cap. After recognition of the AUG eIF2 hydrolyses GTP, regulated by eIF1 and eIF5 (Unbehaun et al. 2004, Hinnebusch 2006), then most initiation factors dissociate and subunit joining takes place with the help of eIF5 and 5B (Unbehaun et al. 2004), resulting in the formation of the 80S ribosome and beginning of elongation.

Several alternative modes of translation initiation exists in eukaryotic cells, such as prokaryotic Shine-Dalgarno-like sequences in mitochondria and chloroplasts, ribosome shunting upon infection by adenoviruses (Yueh et al. 1996) or internal ribosome entry by picornaviruses (Jang et al. 1988) and other viral and cellular mRNAs.

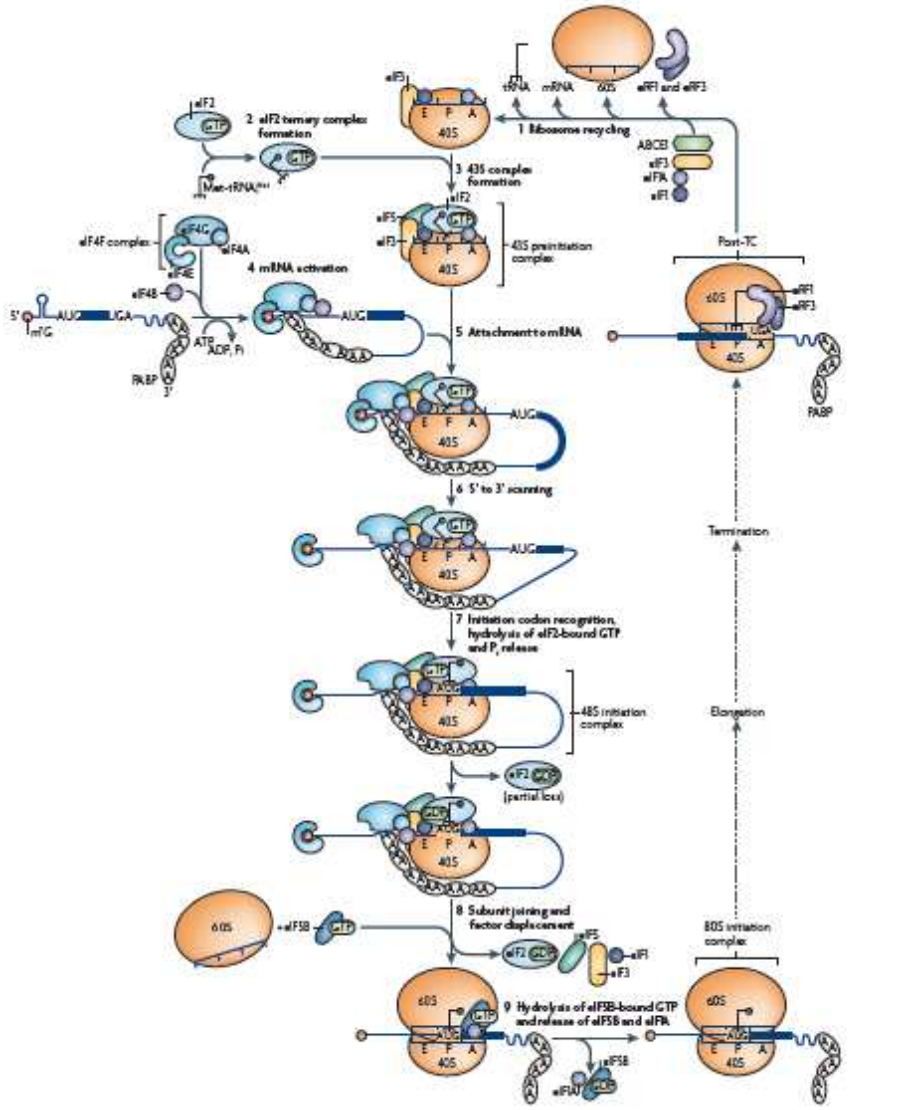

Figure 2: Scheme of cap-dependent translation. Picture from Jackson et al. 2010

IRES-dependent translation initiation

Picornaviruses and several other (+) strand RNA viruses e.g. Hepatitis C virus (HCV) translate via an internal ribosome entry site (IRES), a highly structured element in the 5'-untranslated region (UTR) of the virus RNA that allows ribosomes to bind and commence translation without the 5'-7-methyl-guanosin cap, m^7cap (Pelletier & Sonenberg 1988, Jang et al. 1990, Chen & Sarnow 1995). This mechanism of translation initiation can be found in almost all classes of (+) strand RNA viruses, even in plant viruses (Dorokhov et al.2002, Dreher & Miller 2006).

IRESs differ in their sequence even within types, but secondary structures allow grouping them (Pilipenko et al. 1989). To date there are four different types of viral IRESs (Martinez-Salas et al. 2008, Filbin & Kieft 2009, Hellen 2009) with very different requirements for initiation factors. These differ from m^7caps as well as from each other, e.g. the Type 4 cricket paralysis virus intergenic (IGN) IRES (Wilson et al. 2000a) does not require any factors (Wilson et al. 2000b, Pfingsten et al. 2006, Schüler et al. 2006), while the Type 1 PV IRES needs all initiation factors, except for eIF4E (Pestova et al. 1996), plus additional IRES trans-acting factors (ITAFs) (Hellen & Sarnow 2001, Pacheco et al. 2008).

The IRES elements of picornaviruses consist of 450 to 900 nucleotides (nts) and form five (Cardioviruses) or six (Enteroviruses) stem loops (Nicholson et al. 1991, Jackson et al. 2010). Each stem loop is subject to binding by different proteins, either sequence or structure dependent (Jang et al. 1990). Stem loop I, the clover leaf, is not part of the IRES but necessary for viral replication and with only minimal influence on viral translation (Simoes & Sarnow 1991).

The structure of IRESs and RNA-RNA interactions between stem loops is necessary for translation initiation on IRESs (Ramos et al. 1999, Martinez-Salas 2008), especially on IRESs that do not require protein factors, like the CrPV IGN IRES (Wilson et al. 2000a).

Common to all picornaviral IRESs is a polypyrimidine stretch at their 3' end some 20 nt upstream of the initiator codon AUG (Belsham & Sonenberg 1996, Jang 2006), which is crucial for translation (Nicholson et al. 1991). Type I IRESs, such as the poliovirus IRES, direct the ribosome to a first AUG downstream of the IRES (Pelletier et al. 1988), from which it scans along the RNA until it reaches a second AUG where translation is initiated (Belsham 1992, Bonnal et al. 2005, Jackson et al. 2010). The reason for this process is still unknown. Type II IRESs, such as the EMCV IRES, initiate translation directly at the first AUG after the polypyrimidine tract, even though several AUGs are present within the IRES (Andreev et al. 2007).

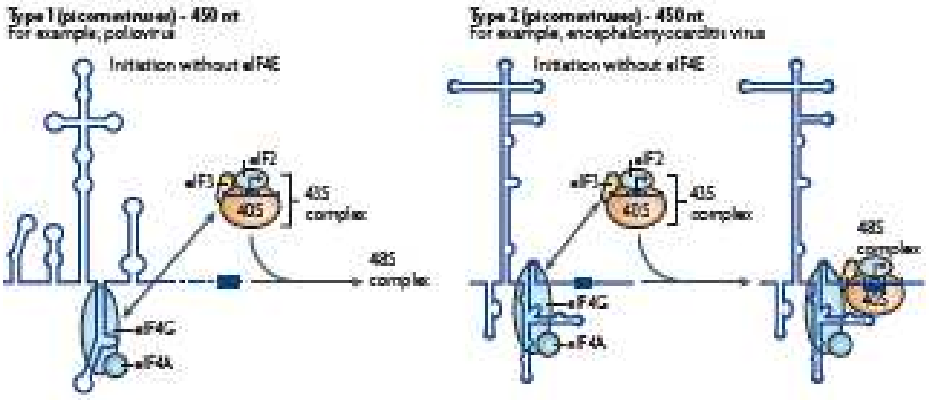

Figure 3: Translation initiation at picornaviral IRES elements type 1 (PV) and type 2 (EMCV). Picture from Jackson et al. 2010

Compared to cap-dependent translation initiation, IRES-dependent translation is inefficient (Andreev et al. 2007), but under certain conditions viral IRESs outperform cap-dependent translation. These conditions are normally induced by viral infection. For example, under high salt conditions IRESs are more efficient than capped RNAs as shown for the Foot-and-mouth-disease virus (FDMV) IRES by Niepmann (2003) and for PV and EMCV by Carrasco and Smith (1976). Some viruses like EMCV and PV change membrane permeability (Aldabe et al. 1996) resulting in an influx of salts, which could decrease cap-dependent translation, but leaves IRES directed translation unaffected or even stimulated (Niepmann 2003, Jünneman et al. 2007).

IRES dependent translation initiation is in most cases independent of the cap-binding complex eIF4F; PV translates even during mitosis when cellular translation is switched off due to phosphorylation of eIF4E binding proteins (eIF4E-BPs) (Hellen & Sarnow 2001). Poliovirus and other picornaviruses take advantage of their independence from the cap binding protein eIF4F, by cleaving its scaffold, eIF4G (Gradi et al. 1998), therefore separating the cap binding part eIF4E from the helicase eIF4A and eIF3 binding part (Ventoso et al. 1998). However, PV still needs eIF4A, eIF3 and the N-terminal part of eIF4G (p100) for efficient translation (Jackson 2005).

IRESs have been identified in the 5' UTR of several cellular proteins (Bernstein et al. 1997, Spriggs et al. 2008), which initiate translation in a way very similar to viral IRESs. Those elements are useful for the cell to keep housekeeping proteins active during stress (apoptosis (Holcik et al. 2003), hypoxia (Lang et al. 2002) or viral infections (Sarnow 1989, Johannes et al. 1999)). Consequently those IRESs are typically found at mRNAs important for the basic

functions of the cells, like X-linked inhibitor of apoptosis (XIAP) (Holcik et al. 2000), the transcription factor c-myc (Martinez-Salas et al. 2001) or the RNA-chaperone unr (Cornelis et al. 2005). An alternative explanation for the existence of cellular IRESs states that they regulate the expression of different isoformal proteins from the same mRNA (Yaman et al. 2003, Bonnal et al. 2005, Tinton et al. 2005).

The function of long cellular 5' UTR as IRES elements was first called in question by several articles indicating cryptic promoters or splice sites in dicistronic constructs (e.g. Baranik et al. 2008) when using DNA transfection. Later Han & Zhang (2002) and Andreev et al. (2009) who used RNA transfection or monocistronic constructs found that cellular IRESs perform very weak compared to viral IRESs. The function of cellular IRESs cannot be eliminated by deleting parts of the IRES (Tinton et al. 2005, Zhang et al. 2010), which can be done with viral IRESs (Nicholson et al. 1991), indicating that cellular IRESs might function differently from viral IRESs (Martinez-Salas et al. 2001) or that their activity might be an experimental artifact (Kozak 2008).

Translation of viral proteins

The RNA genomes of picornaviruses are rather small, due to the limited amount of space in viral particles and the high mutation rate of RNA (Drake & Holland 1999, Shulman et al. 2000). Consequently picornaviruses only possess a limited amount of proteins, which does not include all factors necessary for translation and replication of the viral genome, packing and release of the virus particle. Hence all picornaviruses need to use cellular proteins to aid in translation and replication of their genome. To synthesize viral proteins picornaviruses use canonical translation initiation factors as well as other cellular proteins normally not involved in translation, IRES trans-acting factors, ITAFs (Belsham et al. 2000).

During infection the cellular cap-dependent translation is often suspended by the virus (Etchinson et al. 1984). This leaves initiation factors and ribosomes free to translate exclusively viral proteins, which are not cap-dependent but initiate translation via an internal ribosome entry site, IRES (Jang et al. 1990).

Shut-off of cellular cap-dependent translation can be induced in several ways:

A viral protease (Protease 2A in Poliovirus) cleaves the eukaryotic initiation factor (eIF) 4G and therefore inhibits binding of initiation factors and ribosomes to mRNA (Lamphear et al. 1995, Ohlmann et al. 1995), since the cap-binding complex eIF4F cannot be formed. Dephosphorylation of 4E-BPs (eIF4E binding proteins) after FMDV infections has a similar negative effect on the formation of the eIF4F complex (Gingras et al. 1996) by sequestering eIF4E.

All these mechanisms only take effect after the first rounds of translation of viral proteins are completed. During these first rounds virus RNA competes with cellular mRNAs for factors and ribosomes and may be subject to the inert immune response of the cell.

Several viruses show tissue tropism. They translate, replicate and cause disease in distinct tissues, for example Poliovirus (PV) only causes damage to motor neurons (Johnson 1982). This is due to specific receptors on the cell surface used by the virus (Ren et al. 1990, Morrison et al. 1994), as well as the use of tissue specific cellular proteins for viral translation and replication (Hellen and Sarnow 2001). Transgenic mice expressing the PV receptor CD155 (Koike et al. 1991) ubiquitously, nevertheless only show replication of PV in neurons and muscles (Freistadt et al. 1990, Ren et al. 1992). Upon transfection, circumventing the need for receptors on the cell surface, PV RNA was translated differently in different cell lines, and with highest efficiency in neuronal cells (Bormann et al. 1997).

These studies indicate the use of specific cellular proteins by the IRES, which determine tissue specificity. The factors play a role in canonical cap-dependent translation initiation or origin from a different part of the RNA-metabolism.

Cellular proteins involved in viral translation

Initiation factors

Up to eleven different canonical translation initiation factors (eIF) are thought to be involved in the cap-dependent initiation of translation on cellular mRNAs. For this study the focus is on eIF3, eIF4F and eIF4B since they are shown to bind to IRES elements (Ochs et al. 2002, 2003), and their role in IRES dependent initiation most likely does not differ from their role in cap-dependent initiation. For an overview of initiation factors see table 1 and figure 2.

Initiation factor	Subunits/ molecular weight	Function
eIF1	1 / 13 kDa	Stimulates binding of eIF2 to 40S subunit, inhibits GTP hydrolysis by eIF2
eIF1A	1 / 16 kDa	Stimulates binding of eIF2 to 40S subunit
eIF2	3 / 36, 38, 51 kDa	Binds met-t-RNA and 40 S subunit
eIF2B	5/ 34, 39, 50, 60, 80 kDa	Promotes GDP-GTP exchange on eIF2
eIF3	13 / total app. 750 kDa	Binds other initiation factors and auxiliary proteins, as well as RNA and ribosomal subunits
eIF4A	1 / 46 kDa	RNA-helicase
eIF4B	1 / 70 kDa	Co-factor of eIF4A
eIF4E	1 / 25 kDa	Cap-binding
eIF4F	3 / 25, 46, 170 kDa	Connecting capped RNA to pre-initiation complex, unwinding of RNA secondary structures, consists of eIF4A, 4E and 4G
eIF4G	1/ 170 KDa	Formation of eIF4F with eIF4A and 4E, connecting of eIF3 and PABP to mRNA
eIF4H	1 / 27 kDa	Co-factor of eIF4A
eIF5	1 / 50 kDa	Induces GTP hydrolysis by eIF2
eIF5B	1 / 139 kDa	Subunit joining

Table 1: Canonical initiation factors and their functions in cap-dependent translation initiation (adapted from Jackson et al. 2010).

eIF3

eIF3 is a protein consisting, in mammals, of 11-13 subunits ranging in size from 28 to 170 kDa, which together form a 650-800 kDa complex (LeFebvre et al. 2004, Dong & Zang 2006, Hinnebusch 2006, Masutani et al. 2007). Only six of these subunits seem to be absolutely necessary for translation, p170, p116, p110, p48, p47 and p40, they form the core of eIF3 (Masutani et al. 2007). eIF3 from budding yeast (*S.cerevisiae*) consists of only 6 subunits and is able to function in vitro with only five, however deletion mutants of eIF3 subunits in yeast show a temperature sensitive growth phenotype (Methot et al. 1997). This indicates a

regulatory function for other non-essential subunits in higher eukaryotes, as p40 in sucrose-starved *Arabidopsis thaliana* (Roy et al. 2010) and spore formation in *S. pombe* (Ray et al. 2008). Concurring with these results Masutani et al. (2007) and Hinnebusch (2006) suggest a different composition of core eIF3 for different RNAs or modes of initiation.

eIF3 binds to the 40S ribosomal subunit (Benne & Hershey 1976, Unbehaun et al. 2004) and via eIF4F to the 5' cap (Lefebvre et al. 2004), therefore connecting the ribosomal subunit to the mRNA. It also connects other initiation factors, such as eIF1, eIF2, eIF5 (Phan et al. 1998) and eIF4B in the scanning pre-initiation complex and during subunit joining (Pestova et al. 1998, Jivotovskaya et al. 2005). Its connection to the 40S subunit inhibits premature subunit joining since it masks the binding site for the 60S subunit (Hinnebusch 2006). eIF3 is also involved in ribosome recycling, since it binds to 40S subunits after ribosome disassembly at termination (Kolupaeva et al. 2005) preventing formation of empty ribosomes. Thus, eIF3 is involved in almost every step of translation (Hinnebusch 2006) and serves as a scaffold for other proteins and RNAs, since it does not show any enzymatic activity on its own. Taken this important role of eIF3 it is remarkable that no homologue of eIF3 was found in bacteria (Nielsen et al. 2004).

eIF3 was shown to bind directly to IRES elements (Lopez de Quinto et al. 2001, Siridechadilok et al. 2005). Its RNA binding ability was mapped to subunit p116 (Methot et al. 1997) and p110 (Lopez de Quinto et al. 2001). Even IRESs with a low demand for cellular factors, like HCV, need eIF3 to completely initiate translation (Martinez-Salas et al. 2001).

A different composition of eIF3 for IRES dependent translation as compared to cap-dependent translation is possible (Dong & Zhang 2006), since sequestering the eIF3 subunit p48 by the cellular stress-induced protein p56 did not inhibit IRES dependent translation on the EMCV IRES, but cap-dependent translation (Hui et al. 2003).

eIF4F

eIF4F is a heterotrimeric protein, consisting of 3 distinct subunits, eIF4A, eIF4G and eIF4E (Yoder-Hill et al. 1993). eIF4E is the cap-binding protein (Tahara et al. 1981), that guides the pre-initiation complex to the mRNA. eIF4A is an ATP-dependent RNA helicase belonging to the family of DEAD-box helicases. eIF4G is a scaffold protein that connects eIF4E, eIF4A and eIF3. To initiate translation the eIF4F complex is assembled after eIF4E is phosphorylated by a mitogen activated kinase (Mnk) (Pyronnet et al.1999, Joshi et al. 2010).

eIF4E is the crucial factor in cap-dependent translation, since it is the only protein that binds to the cap and therefore the mRNA (Sonenberg et al. 1978). Formation of eIF4F is highly regulated, eIF4E needs to be phosphorylated before efficient binding can take place

(Pyronnet et al. 1999). This is done by a mitogen activated kinase (Mnk) which is bound to eIF4G and is therefore in close proximity of eIF4E in the eIF4F complex (Knauf et al. 2001). Additionally eIF4E and through it the formation of eIF4F is regulated by eIF4E binding proteins (eIF4E-BP) which compete for binding with eIF4G (Scheper & Proud 2002).

eIF4G is a scaffold protein with no enzymatic activity of its own that is able to bind several other proteins at the same time as well as to bind RNA via an RNA binding domain at the C-terminal end (Wells et al. 1998, Kolupaeva et al. 2003, Dreher & Miller 2006). Before assembly of eIF4F eIF4G is phosphorylated as well as eIF4E (Raught et al. 2000).
During translation initiation eIF4G connects eIF4E and eIF4A to form eIF4F, as well as binding to eIF3 to connect the ribosome to the mRNA (Morino et al. 2000). It also binds the polyA binding protein (PABP) circularizing the mRNA (Tarun et al.1997). Additionally eIF4G is able to interact with several other proteins, e.g. HSP101 (Dreher & Miller 2006) or Mnk (Hinton et al. 2006).
eIF4G has several interaction domains (Figure 4): PABP and eIF4E binding at the N-terminus, two eIF4A binding sites, eIF3 and Mnk at the C-terminus (Wells et al. 1998, Belsham & Sonenberg 2000, Hinton et al. 2006, Marintchev et al. 2009).

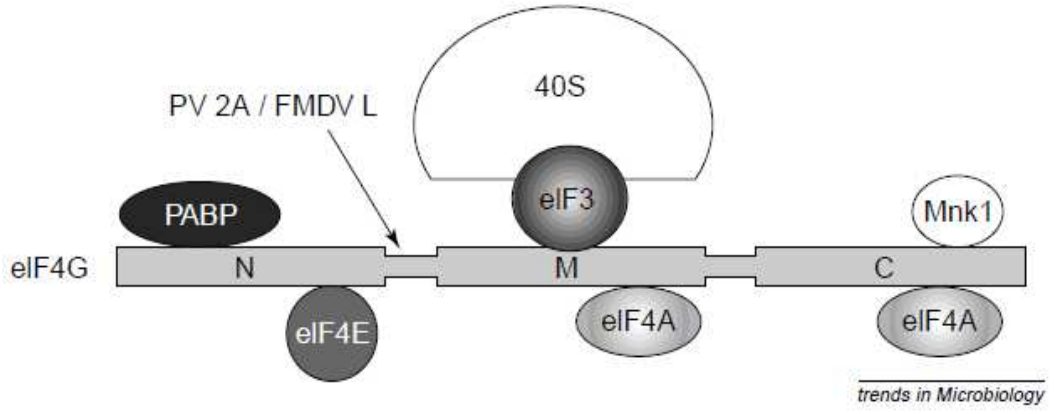

Figure 4: eIF4G with its binding partners, the cutting site for polioviral protease 2A is marked Picture from Belsham & Sonenberg 2000.

Upon infection eIF4G is cleaved by PV protease 2A (Ventoso et al. 1998) and other viral proteases (Figure 4). Only the C-terminal part of eIF4G (p100) is necessary for IRES dependent translation since it binds directly to IRESs (Ohlmann et al. 1995, Ochs et al. 2003,

de Breyne et al. 2006) where it induces conformational changes (Kolupaeva et al. 2003 for EMCV, de Breyne et al. 2006 for PV, Weinlich et al. 2009 for HCV).
At least at the EMCV IRES eIF4G (p100) has an additional function, connecting eIF4A (Lomaki et al. 2000) and eIF3 (Hinton et al. 2006) with each other and the IRES. Together with eIF3 and eIF4A, eIF4G (p100) directs the 40S ribosomal subunit to the RNA resulting in formation of the 48S pre-initiation complex (Hinton et al. 2006).

eIF4A, the last member of the three subunit complex eIF4F, is a DEAD-box RNA helicase (Rogers et al. 1999). It is able to unwind double stranded RNA both in 5'→ 3' and in 3'→5' direction (Rogers et al. 2001). eIF4A is very important during scanning since it unwinds secondary structures in the 5' UTR of mRNAs allowing the pre-initiation complex to move along the mRNA from the cap to the AUG starting codon. eIF4A can act as a RNA-helicase on its own, but binding to eIF4G increases its activity (Marintchev et al. 2009), as does binding of its co-factors eIF4B and eIF4H (Rogers et al. 1999, 2001) or PABP (Bi et al. 2000).
eIF4A was shown to bind directly to IRES elements. It most likely acts as a helicase in IRES-dependent translation as well, opening secondary and tertiary structures to expose or hide binding sites for initiation factors and other proteins (Kolupaeva et al. 2003). eIF4A is absolutely necessary for translation initiation at type I and II IRESs (Pause et al. 1994).

eIF4B

The dimeric protein eIF4B (Methot et al. 1996a) is a co-factor of eIF4A (Altmann et al. 1995, Rogers et al. 2001). It stimulates its helicase activity (Methot et al. 1994) and binding to the IRES (Kolupaeva et al. 2003). Nevertheless eIF4B also possesses a RNA recognition motif and a RNA binding domain of its own (Methot et al. 1994, Rogers et al. 2001) and binds to RNA (Altmann et al. 1995).
eIF4B binds to the PV IRES, more precisely to stem loops IV and V (Ochs et al. 2002). While binding to stem loop V is essential for IRES activity, binding to stem loop IV is not (Ochs et al. 2002). Binding to the IRES by eIF4B may help connecting the ribosome to the IRES, since eIF4B binds 18S rRNA (Methot et al. 1996b) as well as the p170 subunit of eIF3 (Bushell et al. 2001, Ochs et al. 2002). eIF4B connecting to the IRES seems to be sequence specific since even one nucleotide changes severely impairs eIF4B binding to the PV IRES (Ochs et al. 2002, 2003).
Another function of eIF4B in viral translation initiation may be the interaction of eIF4B with PABP (Bushell et al. 2001) and the consequent circularization of mRNA when eIF4G is cleaved and circularization in the normal way (eIF4G-PABP) is impossible.

Taken all this together there is a high redundancy in factor binding since almost every initiation factor interacts with every other initiation factor on some level. Initiation factors not only interact with each other and mutually modulate their functions, but also bind to a variety of cellular proteins (Pyronnet et al. 1999, Bushell et al. 2001, Dreher & Miller 2006).

IRES trans-acting factors (ITAFs)

In some cases, e.g. PV, FMDV or EMDV (encephalomyocarditis virus), more than the canonical factors are necessary for successful initiation of translation on IRES elements. These other factors are cellular proteins not used in cap-dependent initiation but involved in IRES-mediated translation. They are called IRES trans-acting factors (ITAFs). ITAFs are normally involved in other aspects of RNA metabolism, such as transcription, splicing or as chaperones for mRNA. They can form and act as homo- or heterodimers (Pilipenko et al. 2000) and interact with each other as well as with initiation factors and viral proteins.
Some ITAFs are not essential for IRES dependent translation but simply enhance translation efficiency. Nevertheless without them the efficiency of IRES dependent translation initiation would be too low to be physiologically relevant (e.g. Pestova et al. 1996). Some of them are located in the nucleus (Detjen et al. 1978) in uninfected cells, but since infection with PV causes severe damage to the cell, including destruction of nuclear pore complexes and cleavage of nuclear localization signals (NLS) by viral proteases (Shiroki et al. 1999), this leads to a redistribution of factors from the nucleus to the cytoplasm. Different IRESs have different requirements for ITAFs (Pacheco et al. 2008), sometimes even closely related viruses need a different set of ITAFs, leading to tissue tropism (Gromeier et al. 2000) of the virus.

La

La was first identified as an autoantigen in people suffering from systemic lupus erythematosus (Izumi et al. 2004). It contains one highly specific domain, the La-motif and two other classical RNA recognition motifs (Pudi et al. 2003). The La-motif by itself is not able to bind RNA (Izumi et al. 2004) but enhances binding of the other RNA-binding motifs tremendously through its aromatic surface residues (Dong et al. 2004). La is highly abundant in cells (Wollin & Cedervall 2002) and mostly located in the nucleus (Meerovitch et al. 1993).
La plays an important role in PV translation; it was one of the first ITAFs identified (Meerovitch et al. 1989). In vitro experiments with translation are very often conducted in rabbit reticulocyte lysate (RRL), which does not contain significant amounts of La (Meerovitch et al. 1993). Therefore RRL needs to be supplemented with HeLa extract (Kim & Jang 1999)

or purified La (Shiroki et al. 1999) for translation to occur efficiently at the PV IRES. This was the first hint that La is a necessary factor for efficient PV translation.

There are studies indicating binding of La to the PV IRES at stem loop IV (Meerovitch et al. 1989, 1993, Das et al. 1994) and stimulating translation initiation at the PV IRES directly (Craig et al. 1997, Isoyama et al. 1999, Shiroki et al. 1999). In contrast Kim & Jang (1999) showed that La enhances translation initiation at the EMCV IRES elements synergistically with the polypyrimidine tract binding protein (PTB), but not by itself. Moreover La competes with PTB in binding to the EMCV IRES, since binding of La can be inhibited by an excess of PTB, concluding that La is not a necessary ITAF for all (+) RNA viruses (Meerovitch et al. 1993, Kim & Jang 1999).

PAPB PolyA binding protein

Cellular mRNAs as well as some viral RNAs have polyA tails of various lengths (Munroe & Jacobson 1990). These polyA stretches protect the mRNA from degradation (Ross 1995) and are implicated in circularization of mRNAs (Gallie 1991 and figure 5) and consequent regulation of translation.

The polyA binding protein (PABP) binds to these polyA tails, which protects them from nucleases. Since it only needs 8-20 As to bind (Herold et al. 2001, Patel et al. 2005) several PABPs bind to a polyA tail simultaneously. The 72 kDa protein PABP contains four RNA recognition motifs (RRMs) (Grange et al. 1987), a eIF4G binding domain and a proline-rich C-terminal domain responsible for several protein–protein interactions (Deo et al. 2001, Kozlov et al. 2002, Mangus et al. 2003).

PABP stimulates cap-dependent (Kavejian et al. 2005) and IRES-dependent translation (Paulous et al. 2003, Thoma et al. 2004), most likely through the circularization of mRNA (Jackson et al. 2010) via its interaction with eIF4G (Le et al. 1997), eIF4A (Searfoss et al. 2001) or PCBP2 (Wang et al. 1999), which all bind to mRNAs at their 5’ end, therefore their interaction with PABP bridges 5’ and 3’ end of mRNAs (Jackson et al. 2010). Stimulation of translation by PABP still occurs when eIF4G is cleaved due to PV or FMDV infection (Thoma et al. 2004) or even when a single point mutation destroys the affinity of PABP for eIF4G (Tarun et al. 1997, Kavejian et al. 2005), rendering circularization via eIF4G-PABP binding impossible (Searfoss et al. 2001). However since PABP also interacts with eIF4A and PCBP2, which bind to the IRES as well, circularization may still occur (Alvarez et al. 2005).

Upon PV infection PABP is cleaved between the RNA binding domains and the protein interacting C-terminus by the viral proteases 2A (Joachims et al. 1999, Kerekatte et al. 1999,

Deo et al. 2001) and 3C (Herold et al. 2001, Kuyumcu-Martinez et al. 2002). However viral proteases never completely degrade PABP during infection (Kuyumcu-Martinez et al. 2002).
In bloodcells, PABP, together with the poly-r(C) binding protein (PCBP2), forms an important part of the α-globin stability complex that protects RNA from deadenylation by RNases (Weiss et al. 1995, Wang et al. 1999). It is therefore present in RRL.

PCBP2 Poly-r(C) binding protein

PCBP2 was identified as hnRNP E (heterogeneous ribonucleoprotein E), a nuclear protein that binds to poly(C) stretches (Leffers et al. 1995). Together with PABP it is part of the α-globin stability complex (Weiss et al. 1995, Kiledijan et al. 1995, Wang et al. 1999) and is therefore present in RRL (Makeyev et al. 2002). Structurally PCBP2 consists of 3 KH domains (hnRNP K homology) connected by flexible linkers (Blyn et al. 1995, Leffers et al. 1995, Sean et al. 2008). Only KH1 is able to bind to the PV 5' UTR (Silvera et al. 1999, Spear et al. 2008) by itself, inhibiting translation rather than stimulating it (Spear et al. 2008).
PCBP2 plays an important role in the switch from PV translation to replication. It is able to bind at two sites in the PV 5' UTR, the clover leaf (stem loop I) and stem loop IV in the IRES. Its affinity to the clover leaf structure is low in early stages of infection, but increases in later stages due to formation of a ternary complex together with the viral protein 3CD (Parsley et al. 1997, Spear et al. 2008) and cleavage of KH domain 3, which is necessary for binding of stem loop IV but not stem loop I (Walter et al. 2002, Perera et al. 2007). The ternary complex of PCBP2, stem loop I and 3CD initiates replication by the viral polymerase 3C from the 3' end of the PV RNA (Barton et al. 1999), maybe through interaction with PABP (Wang et al. 1999, Herold et al. 2001). During early stages of infection PCBP2 binds to stem loop IV of the IRES with high affinity (Sean et al. 2008) and is thought to enhance translation by recruiting ribosomes to the AUG starting codon.
The binding site of PCBP2 at the PV IRES was mapped in more detail to the top of stem loop IV, especially a C-rich region around nt 332 in a loop structure (Gamarnik et al. 2000). Addition of PCBP2 to in vitro translation reaction in RRL stimulates translation initiated at the PV IRES and other type 1 picornaviral IRESs, but not type 2 IRESs like EMCV (Sean et al. 2008).

PTB Polypyrimidine tract binding protein

PTB was the first ITAF identified to bind to the PV IRES (Hellen et al. 1993), more precisely to a bulge structure in stem loop V (Ochs et al. 2002). It was first identified as another hnRNP, hnRNP I, which is, in uninfected cells, located in the nucleus and involved in pre-mRNA

processing (Ghetti et al. 1992). It binds indiscriminately to polypyrimidine stretches which are an important feature of picornaviral IRESs (Jackson & Kaminski 1995) as well as introns (Padgett et al. 1986). By binding to polypyrimidine stretches of pre-mRNA PTB masks alternative splice sites and suppresses splice variants (Perez et al. 1997, Sharma et al. 2005). PTB contains a total of four RNA recognition motifs (RRMs). Two are located in its C-terminus (Bothwell et al. 1991, Gosert et al. 2000) and two weaker ones in the N-terminus. .

PTB enhances translation on the PV and HAV IRESs in cells (Gosert et al. 2000) by stabilizing IRES structure, essentially acting as a chaperone for the PV IRES (Niepmann et al. 1997, Song et al. 2005, Pacheco & Martinez-Salas 2010).

PTB is present in RRL. Depletion of PTB from RRL severely decreased translation efficiency on the PV and EMCV IRES (Kim & Jang 1999), but addition of un-physiological amounts (> 5 µg/ml) of recombinant PTB decreased translation as well. There seems to be a window of acceptable concentrations. Addition of La (Kim & Jang 1999) but not unr (Hunt et al. 1998) diminished the effect of PTB at un-physiological concentrations, therefore confirming the fact that some factor in HeLa extract enhances binding of PTB to RNA (Oh et al. 1998).

Unr

Unr (upstream of n-ras) (Jeffers et al. 1990) is a chaperone with five cold shock domains, which are basically nucleic acid binding domains (Jacquemin-Sablon et al. 1994). In uninfected cells unr serves as a scaffold protein protecting mRNAs and as a regulator of apoptosis (Dormoy-Raclet et al. 2007). It binds to single stranded and purin-rich RNAs, but not to polyA stretches or tails (Jacquemin-Sablon et al. 1994, Triqueneaux et al. 1999, Patel et al. 2005).

Unr is expressed ubiquitously in the cytoplasm (Jeffers et al. 1990, Jacquemin-Sablon et al. 1994). However, its concentration in RRL is rather low (Hunt et al. 1998, Dormoy-Raclet et al. 2005).

Unr is always in close contact with its binding partner unrip (unr interacting protein, 38kDa) (Hunt et al. 1998), which is implied to be involved in assembly of snRNPs (Carissimi et al. 2005).

ITAF	Cellular function(s)	Binding to PV IRES	Stimulation of PV IRES	Stimulates IRESs	No effect on IRES
PTB	Splice regulation	+	+	HAV HCV HRV FMDV (EMCV)	Cellular IRESs TMEV
La	Chaperon	+	+	HCV HRV cellular IRESs	EMCV
PCBP2	Chaperon/ translational regulator	+	+	HAV HCV HRV	EMCV FMDV
PABP	Chaperon/enhancer	-	+	HAV Cellular IRESs	
unr	Chaperon	+	+ (-)	HRV cellular IRES	EMCV FMDV
DAP5	Scaffold			cellular IRESs	EMCV
DHX29	Helicase			cellular IRESs	
GAPDH	Glycolysis			HAV	
Gemin5	snRNP assembly			FMDV HCV	
hnRNP A	Shuttling, splicing, telomeric capping			HCV HRV	EMCV FMDV Cellular IRESs
hnRNP C	Shuttling, splicing			HCV cellular IRESs	
hnRNP D	Shuttling			HCV	Cellular IRESs
hnRNP K	Chaperon				
hnRNP L	Splicing			HCV	
HSP101	Chaperon			TMV	
$ITAF_{45}$/EpB1	Transcription			FMDV	EMCV
nucleolin	Chaperon/Helicase	-	+	FMDV HCV	
SRp20	Splicing	-	+		
unrip	snRNP assembly	-	+		

Table 2: Identified IRES trans-acting factors (ITAFs) from the literature cited in the text

Other potential ITAFs

All ITAFs I mentioned so far are well studied and proven to interact with at least one kind of picornaviral IRES apart from the PV IRES in multiple studies. They are also expressed almost ubiquitously and none of them can be the sole reason for the tissue tropism of PV.

However, there are many other potential ITAFs (Pacheco & Martinez-Salas 2010), which are not well studied or their role in IRES dependent translation has only been discovered recently (table 2).

- There is for example $ITAF_{45}$/EpB1 which together with PTB is necessary for translation on the FMDV IRES (Pillipenko et al. 2000, Monie et al. 2007) and interacts with the EMCV IRES (Bonnal et al. 2005).
- Heterogenous nuclear riboproteins (hnRNPs), a class of at least 20 proteins, are involved in maturation of mRNA, export from the nucleus and protection from nucleases (Mili et al. 2001). Some of them are already well established as ITAFs, such as PTB and PCBP2 (hnRNP E and I), but several more may be involved in IRES-dependent translation initiation. Cellular IRESs are often regulated by hnRNPs. hnRNP A is a negative regulator of the cellular IRES in the XIAP (X-linked inhibitor of apoptosis) mRNA (Lewis et al. 2007) and capped mRNAs (Svitkin et al. 1996). Regarding translation initiation at viral IRESs, hnRNP A was shown to stimulate translation at the HRV (Cammas et al. 2007) but not FMDV (Pacheco et al. 2008) IRES.
- Another nuclear protein implicated as a potential ITAF is nucleolin, the major protein in the nucleolus, the place of transcription and assembly of ribosomal RNAs (Ginisty et al. 1999). However, during PV and CBV3 infection it relocates to the cytoplasm (Waggoner & Sarnow 1998, Rassmann et al. 2006). Nucleolin binds to the 3' UTR of PV and enhances translation and replication (Waggoner & Sarnow 1998) However, the PV 3' UTR shows significant effects on translation controlled by the PV and HRV IRES (Dobrikova et al. 2003). Nucleolin was also identified to bind to the FMDV IRES (Pacheco et al. 2008), but no functional tests were performed.
- Proteins which are partial homologues of initiations factors, such as DAP5 (Imataka et al. 1997), a homologue of eIF4G (Dormoy-Raclet et al. 2005), or DHX 29, another DEAD box helicase (Jackson et al. 2010), interact with IRESs and cellular mRNAs (Hundsdoerfer et al. 2005) to enhance translation efficiency and may functionally replace initiation factors. Late in HCV infection the function of eIF4A is taken by the viral helicase NS3 (Du et al. 2002).
- Gemin5 is in uninfected cells involved in snRNP assembly as part of the survival of motor neurons complex (Battle et al. 2006). It binds to the IRES of FMDV (Pacheco et al. 2008) and

HCV (Pacheco et al. 2009) and inhibits translation in both cases (Pacheco et al. 2009). Binding to the PV IRES was not shown yet, but its specific binding motif AUUUUUG (Battle et al. 2006) can be found in the PV IRES.

Another way proteins could act as ITAFs is by modulating binding of initiation factors and known ITAFs to the IRES, e.g. SRp20 interacts with the KH3 domain of PCBP2 and is necessary for stimulation of PV IRES translation by PCBP2 (Bedard et al. 2007) without binding to the IRES. Other proteins could have similar modulating functions, maybe in addition to their RNA binding.

Interaction of ITAFs and initiation factors

Effects of an ITAF or initiation factor on the IRES may depend on their interaction partners, the cellular localization or position in the cell cycle, and can be diametrical (Pacheco & Martinez-Salas 2010).

Proteins binding to IRES elements interact firstly with RNA, the IRES. But they also interact with each other and enhance or inhibit binding of other proteins to the IRES. Sometimes the whole is more than the sum of its parts, e.g. PTB, unr and PCBP2 act synergistically on the HRV IRES (Hunt et al. 1998).

However, negative regulation occurs as well. La binding to the EMCV IRES is inhibited by PTB at physiological salt concentrations (Kim & Jang 1999). Additionally, upon binding of factors and under changing salt conditions, IRESs undergo structural changes (Martinez-Salas et al. 2001), exposing or hiding potential binding sites for other factors.

Some ITAFs interact with each other during their cellular function, e.g. PCBP2 and PABP are both part of the α-globin mRNA stability complex (Wang et al. 1999), and unr and PABP interact on the autoregulatory sequence of PABP mRNA (Patel et al. 2005). La stimulates binding of PABP to polyA tails (Svitkin et al. 2009).

Protein-protein interactions that circularize mRNAs, PABP with eIF4G (Kavejian et al. 2005), with eIF4B (Bushell et al. 2001) and others are very important for cellular as well as viral mRNAs and will be explained in detail in the next chapter.

Circularization of mRNAs

mRNAs are thought to be circular (Wells et al. 1998, Prevot ez al. 2003). There are several mechanisms for mRNAs to achieve circularization (figure 5).

By far the most common one is the interaction of the cap structure and the polyA tail via interaction of the polyA-binding protein (PABP) and eIF4G (Le et al. 1997, Jackson et al. 2010). The function of PABP can be taken by other proteins, such as SLIP1, which connects eIF4G to the 3' UTR of histone mRNA (Cakmakci et al. 2008). HCV, a virus without polyA tail, achieves circularization via dimerisation of IGF2bp which binds sequences in the 5' and 3' UTR (Weinlich et al. 2009). Other flaviviruses, e.g. Dengue virus, use direct base pairing between complementary sequences in the 5' and 3' UTR (Alvarez et al. 2005) completely circumventing the need for proteins.

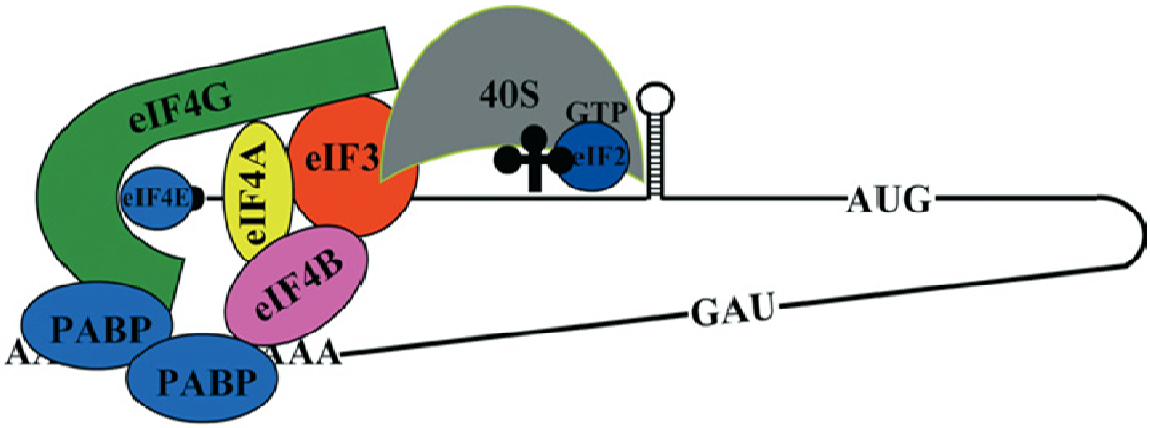

Figure 5: Circularization of mRNA via interaction of PABP with eIF4G and eIF4B. Picture from Spriggs et al. 2008.

The reasons for circularization of mRNA are not fully explained yet. We know that circular RNAs are protected from exonucleases and that circularization enhances translation (Munroe & Jacobson 1990, Gallie 1991).

There are two theories on the mechanism of enhancement:

By circularization, the end of the mRNA comes in close proximity to the cap, so that terminating ribosomes form a high local ribosome density and immediate re-initiation becomes easier (Mangus et al. 2003, Bradrick et al. 2006, Lloyd 2006). This theory is especially interesting since stimulation by polyA tails is observed strongest in depleted extract under highly competitive circumstances (Gallie 1991).The proximity of cap and 3' end of the coding region also facilitates reconnection of eIF4F if it loses contact with the cap (Jackson et al. 2010) during initiation.

Circularization may be a signal to the cap via the eIF4G-PABP interaction that the RNA is not degraded at the 3' end and translation can start, since translation of truncated mRNAs would

lead to non-functional proteins. Binding of PABP leads to conformational changes of eIF4G and eIF4E resulting in enhanced cap-binding by eIF4E (Borman et al. 2000). Addition of PABP enhances formation of 48S complexes at the CBV3 IRES (Bradrick et al. 2007) and at capped RNA (Kahvejian et al. 2005), indicating a role for PABP and polyA tails at the level of translation initiation.
During PV infection PABP and eIF4G are cleaved (Deo et al. 2001), and PABP is no longer able to connect to viral RNA via eIF4G (Hellen & Sarnow 2001). This may open the loop and act as a signal to stop translation and commence replication.

microRNAs

Since their discovery in the early 90s (Lee et al. 1993) microRNAs (miRNAs) were shown to be involved in almost any kind of gene regulation (e.g. Cannell et al. 2008, Kozak 2008). miRNAs are rather conserved (Friedman et al. 2008), for most of them homologues can be found between humans and mice (Lagos-Quintana et al. 2003) or even *C. elegans* and *D. melanogaster* (van Rji et al. 2006). miRNAs or at least small regulatory RNAs have even been identified in archea and bacteria (Repoila et al. 2003, Berghoff et al. 2009) and in the genomes of viruses (Bogerd et al. 2010). miRNAs bind to their target mRNA, in most cases, at the 3' UTR (Yekta et al. 2004). One specific miRNA can have several 100 target mRNAs (Friedman et al. 2009), and one mRNA can be targeted by several different miRNAs (Farh et al. 2005).
Regarding the abundance and importance of miRNAs in cells it is very likely that viruses as well as cellular mRNAs are targeted by miRNAs, either as part of the innate immune response (Bagasra & Prilliman 2004, Baulcombe 2004, Lecellier et al. 2005, van Rji et al. 2006, Pedersen et al. 2007) or employed by the virus to enhance their translation (Henke, Goergen et al. 2008) or replication efficiency (Jopling et al. 2005).

There are two different modes of action for miRNAs. Either the siRNA pathway, where mRNAs are marked by perfect binding of small RNAs for degradation (Bernstein et al. 2001, Hauser et al. 2009) or the miRNA pathway, where translation of mRNAs is inhibited (Kozlov et al. 2010) or stimulated (Orom et al. 2008) by the binding of a small part of the miRNA, the seed sequence (Petersen et al. 2006, Henke, Goergen et al. 2008). Incomplete binding of the miRNA to its mRNA target is the main difference between the miRNA pathway and the siRNA pathway (Kelly & Russel 2009), where the entire sequence of the siRNA binds to the mRNA, inducing the RNA-induced silencing complex (RISC) via double stranded RNA (Hammond et al. 2001, Rhoades et al. 2002).

In cells miRNAs are transcribed in the nucleus (figure 6) from intronic or intergenic regions as pri-miRNA by RNA-Polymerase II. Pri-miRNAs can be up to several thousand nucleotides long (Jones-Rhoads et al. 2006) and contain one or more imperfect 80 nt stem loops, which are subsequently cleaved off by the RNase Drosha (Gregory et al. 2005, Lagos-Quintana et al. 2002, 2003). The resulting short stem loops (pre-miRNAs) are then exported into the cytoplasm by exportin 5 (Kai et al. 2010). Here they are further processed by another RNase, DICER, which cleaves the loop, leading to a double stranded RNA intermediate, which is introduced into the RNA-induced silencing complex (RISC) and separated into two short single stranded RNAs (Bartel 2009). One is the mature miRNA, the active strand or guide strand, which stays in the RISC (Schwarz et al. 2003, Wang et al. 2008). The other one is named the passenger strand and is usually discarded and degraded (Hutvagner et al. 2001, Wang et al. 2008). In some cases both strands can act as a mature miRNA, like miRNA 422a. In this case incorporation into RISC seems to be a stochastic process (Schwarz et al. 2003). Choice of the guide strand is dependent on strength of 5' pairing of the double stranded intermediate (Schwarz et al. 2003).

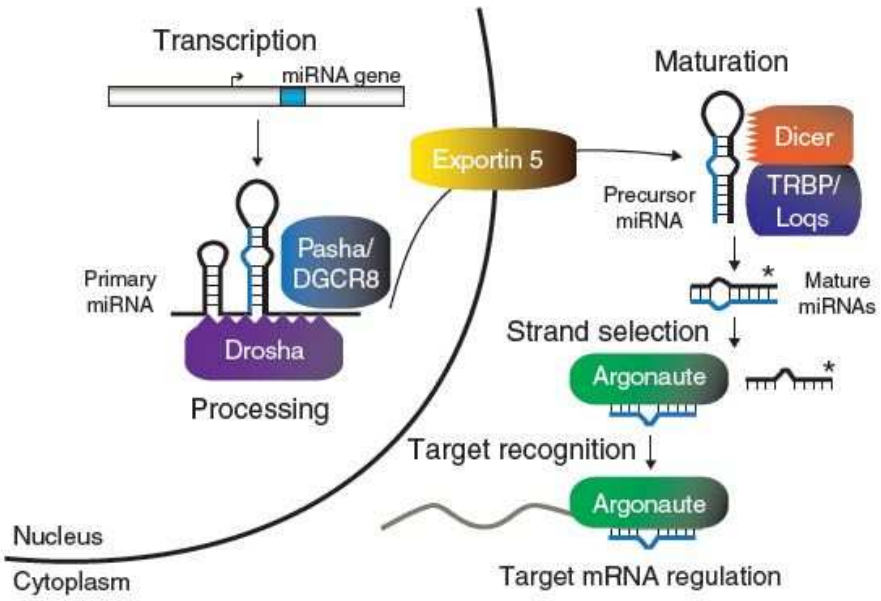

Figure 6: Biosynthesis of miRNA and formation of RISC. Picture from Kai & Pasquinelli 2010.

The seed sequence of miRNAs, usually nucleotides 2 - 8, pairs in a Watson-Crick manner with the 3' UTR of mRNAs (Nielsen et al. 2007, Bartel 2009). The remainder of the miRNA serves as binding partners for argonaut proteins as part of RISC that further process the mRNA (e.g. Hammond et al. 2001). There are several possibilities what happens to the

mRNA after binding of miRNAs (Zinovyev et al. 2010). It is either degraded (Yekta et al. 2004, Fabian et al. 2009), stored in P-bodies, thus delaying translation of a particular mRNA (Pillai et al. 2007, Kozlov et al. 2010), or ribosomes are stalled (Nottrot et al. 2006, figure 7).

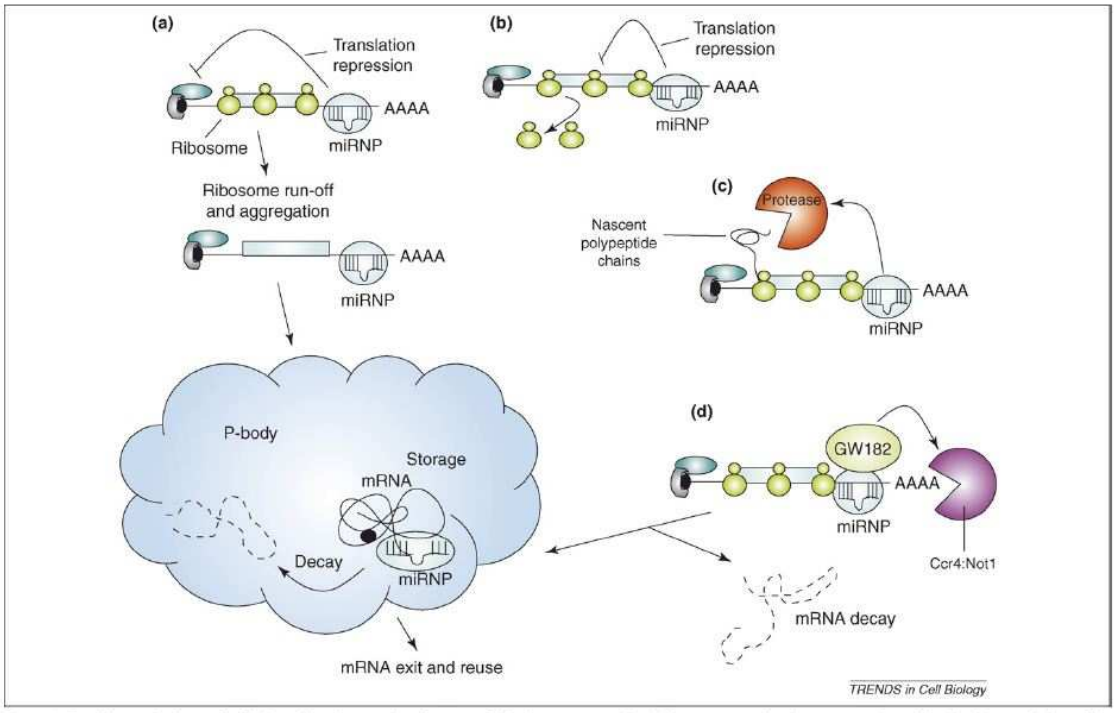

Figure 7: Possible mechanisms for miRNA induced translation repression. Picture from Pillai et al. 2007.

There are several theories regarding the seed sequence necessary for miRNA binding leading to effects. The consensus at the moment is that at least 6 nt need to pair in a Watson-Crick manner. The target site should be in an AU rich environment (Sun et al. 2010) and should not be in a highly structured area or too close to the polyA tail and the translation termination signal (Bartel 2009, Hauser et al. 2009).

Some miRNAs are expressed ubiquitously, but other show very strong tissue specificity, e.g. miRNA-122 is almost exclusively expressed in liver tissue (Lagos-Quintana et al. 2002), while miRNA-21 is expressed everywhere, except in neuronal tissue (Lagos-Quintana et al. 2003). The specificity is part of regulatory potential of miRNAs (Kelly & Russel 2009). miRNA expression changes during development (Lagos-Quintana et al. 2001) and under stress (Sunkar & Zhu 2004) which led to the original name small temporal RNAs (stRNA). Especially the distribution of miRNAs in neuronal tissue is studied, since they are implicated in regulation

of neuron proliferation and axon movement (Landgraf et al. 2007). Several miRNAs were identified in high through-put screens to be brain-specific, they can even be mapped directly to one brain region, e.g. let 7c is mainly expressed in the midbrain (Lagos-Quintana et al. 2002).

Regarding the ubiquitous distribution and strong regulation of miRNAs in neuronal cells, it is possible that one or more miRNAs act as ITAFs for the PV IRES, either stimulating or inhibiting translation initiation.

Indirect effects of miRNA on PV IRES dependent translation may also occur via interaction of miRNAs with known ITAFs, for example alternative splicing of PTB is regulated by the neuronal miRNA-124 (Makeyev et al. 2007) and miRNA-326.

Aim of this study

Poliovirus translation and replication can occur in neuronal cells where it causes degeneration and lysis of cells and consequent irreversible paralysis of the infected person. Other cell types are much less affected by PV infection and do not support translation of the virus as well. The reasons for the tissue preference of poliovirus may be found in its translation via an internal ribosome entry site (IRES), which in addition to cellular initiation factors uses proteins normally not involved in translation to achieve efficient translation. Several such factors are known, but neither do these known factors explain the tissue specificity of poliovirus, nor are they sufficient to initiate translation at the PV IRES using only purified components. Additional factors must be present in cells, especially in neuronal cells, that are necessary for translation initiation on the PV IRES. The goal of my PhD work was, to identify those factors as well as additional roles for and interactions of known factors. Thus I tried to gain further insights in the mechanisms of translation initiation at the prototypical picornavirus IRES of Poliovirus and the tissue specificity of the virus.

Materials and Methods

Purification of eukaryotic initiation factors from Rabbit Reticulocyte Lysate (RRL)

Several purification methods were tested, but did not yield the desired result, pure initiation factors. The method described here was established by Prof. Shatsky in his laboratory at the Belozersky Institute for Physio-Chemical Biology at the Moscow State University and performed there.

Buffers

Resuspension buffer		Buffer A	
20 mM	Tris-HCl (pH 7.6)	20 mM	Tris-HCl (pH 7.6)
0.1 mM	EDTA	0.1 mM	EDTA
1 mM	DTT	1 mM	DTT
10 mM	KCl	10 %	Glycerol
0.25 mM	Sucrose	KCl (50, 100, 300, 400, 500,1000 mM)	
1 mM	$MgCl_2$		

Column materials

DEAE (DE-52)	Whatmann
Phosphocellulose (PC-11)	Whatmann
Cap-Sepharose	Roche
MonoQ	GE-Healthcare

Determination of protein concentration using absorbance

Protein concentration was determined by measuring the absorbance of light with a wavelength of 280 nm (OD_{280}) by the solution. The protein solution was filled into a 1 ml UV-permeable cuvette and absorbance was detected using a photometer. Protein concentration was estimated using a formula derived from the Lambert-Beer law:

Concentration (mg/ml) = Absorbance / path length (cm).

Since with the path length was 1 cm in this photometer the measured absorbance roughly equals the protein concentration.

The specific absorption coefficient found in the Lambert-Beer law is set to one, since it differs widely between different proteins and in a mixture of proteins.
Highly concentrated protein solutions were diluted 1:10.

Preparation of ribosomal salt wash (RSW)

RRL was centrifuged at 40'000 rpm (rotor Ti45 in a Beckman ultracentrifuge) for 4 h, the supernatant was discarded (or stored at -80 ℃ for further use) and the pellets containing the ribosomes were resuspended in resuspension buffer.
500 mM $MgCl_2$ was added to a final concentration of 3 mM. The KCl concentration of the resuspended ribosomes was adjusted with 3 M KCl to 500 mM KCl. The high concentration of salts dissociates proteins from the ribosomes but leaves the ribosomal subunits intact.
By centrifugation at 41'000 rpm (Ti45) for 4 h ribosomes were pelleted while the dissociated proteins remained in the supernatant. The supernatant after this step is the ribosomal salt wash (RSW).

Ammonium sulfate precipitation

Solid ammonium sulfate (calculated using table in appendix) was added slowly to the RSW at 4 ℃ with continuous stirring. Precipitated protein s were pelleted at 10'000 rpm for 30 min at 4 ℃ in a Beckmann centrifuge (rotor JA-20). 40 %, 40 -50 % and 50-70 % fractions were obtained. Ammonium sulfate precipitates can be stored at 4 ℃ and were used to transport RSW to Moscow.

Ion-exchange chromatography

The 40 % ammonium sulfate precipitates were dissolved in A-100 and dialyzed against A-50 over night at 4 ℃.
After short centrifugation the cleared solution was applied to a DEAE sepharose column equilibrated with A-100 using a peristaltic pump with a flow rate of 2 ml /min. The column was washed with A-100 until the OD_{280} reached baseline. Buffer was switched to A-300 to elute proteins. 8 ml fractions were collected until the OD_{280} reached baseline.
The fractions containing most protein (cut-off OD_{280} = 1) were pooled, diluted 1:3 with A-0 and applied to a PC column, equilibrated with A-100, using a peristaltic pump with a flow rate of 2 ml/min. The column was washed with A-100 until the OD_{280} reached baseline. Then proteins were eluted using A-400. 8 ml fractions were collected until the OD_{280} reached baseline.
The fractions of the A-400 elution containing most protein (cut-off OD_{280} = 0.6) were precipitated with 50 % ammonium sulfate, resuspended in A-500a and dialyzed over night

against A-500a. Precipitation and dialysis remove glycerol as well as concentrate the proteins.

Sucrose gradients

Sucrose solutions from 5 % (w/w) to 20 % (w/w) were prepared in A-500a and four 28 ml gradients were poured using a gradient mixer. 3.2 ml of the dialyzed protein preparation were pipetted on top of each gradient and the gradients were centrifuged for 32 h at 24'000 rpm (rotor SW 27) in a Beckmann ultracentrifuge. Gradients were then fractionated (1.2 ml fractions) and the OD_{280} measured. Two peaks can be identified, the first one containing eIF3 while the second one contains eIF4F and 4B.

Ion-exchange chromatography

Fractions from sucrose gradients containing eIF3 were pooled and applied to a MonoQ column equilibrated with A-0 on an Äkta FPLC-machine. The column was eluted with a gradient of KCl from 0 mM to 500 mM and peak fractions were tested using SDS-PAGE. This resulted in the elution of pure eIF3.

Cap-Sepharose

Fractions from sucrose gradients containing eIF4F and 4B were pooled and precipitated with concentrated ammonium sulfate solution. The ammonium sulfate precipitate was resuspended in A-100 containing 1 mM Mg-acetate and applied to a Cap-Sepharose column equilibrated with A-100 containing 1 mM Mg-acetate. To ensure binding of eIF4F via its cap-binding moiety eIF4E to the column the flow-through was reapplied to the column twice. The column was washed with 6 column volumes of A-100 containing 1 mM Mg-acetate and 100 µM GTP. Pure eIF4F was eluted with 100 µM m^7GTP in A-100 containing 1 mM Mg-acetate and then dialyzed against A-100 over night at 4 ℃.
eIF4B can be obtained from the flow through via ion-exchange chromatography or may be prepared recombinantly.

Purification of recombinant proteins

Columns

Phenyl-Sepharose	GE Healthcare
MonoQ	GE Healthcare
MonoS	GE Healthcare

Ni-NTA	Qiagen
Talon beads	BD-Bioscience

Buffers

Standard lysis buffer

20 mM	Tris-HCl (pH 7.5)
300 mM	NaCl
0.1 mM	EDTA

Standard washing buffer

20 mM	Tris-HCl (pH 7.5)

PCBP2 lysis buffer

20 mM	Tris-HCl (pH 7.9)
250 mM	NaCl
60 mM	Imidazol
10 %	Glycerol

PCBP2 elution buffer

20 mM	Tris-HCl (pH 7.9)
250 mM	NaCl
200 mM	Imidazol
10 %	Glycerol

Unr lysis buffer

20 mM	Tris-HCl (pH 8)
100 mM	NaCl
10 %	Glycerol

unr resuspension buffers

20 mM	Tris-HCl (pH 8)
150 mM	NaCl
10 %	Glycerol
1 mM	Beta-mercaptoethanol

Urea (8.5 M, 6 M, 4 M, 3 M, 2 M, 1 M)

Buffer for separating gel

375 mM	Tris-HCl (pH 8.8)
0.1 %	SDS

Buffer for stacking gel

125 mM	Tris-HCl (pH 6.8)
0.1 %	SDS

Tris-Glycine buffer

25 mM	Tris-HCl (pH 8.4)
192 mM	Glycin
1.66 mM	DTT

Protein loading buffer

350 mM	Tris (pH 6.8)
0.1 %	SDS
0.12 %	Bromphenolblue
30 %	Glycerin
1 %	β-Mercaptoethanol

Coomassie stain		Destaining solution	
50 %	Ethanol	5 %	Ethanol
10 %	Acetic acid	7.5 %	Acetic acid
0.12 %	Coomassie brilliant blue		

Silver stain solution (SSS) 1		Silver stain solution (SSS) 2	
5 mM	Na_2CO_3	10 mM	$AgNO_3$
4.5 mM	$K_3(FeCN)_6$		
20 mM	$Na_2S_2O_3$		

Silver stain solution (SSS) 3	
2.5 %	Na_2CO_3
0.4 %	formaline

Cathode buffer (western blot)		Anode buffer 1 (western blot)	
25 mM	Tris-HCl (pH 9,4)	0.3 M	Tris-HCl (pH 10.4)
40 mM	Glycin	10 %	Methanol
10 %	Methanol		

Anode buffer 2 (western blot)		TBS-T	
25 mM	Tris-HCl (pH 10.4)	10 mM	Tris-HCl (pH 8,0)
10 %	Methanol	150 mM	NaCl
		0.1 %	Tween 20

Plasmids

All recombinant proteins are N-terminally His-tagged.

pCITE PV1 VP1-2A	from T. Skern
pET15b (eIF4A)	from I. Shatsky
pET15b (eIF4A R362Q)	from I. Shatsky
pET15b (eIF4B)	from I. Shatsky
pET15b (p100)	from I. Shatsky
pET22b PCBP2	from B. Semler
pLaGST	from A. Bindereif
pRSET-unr	from H. Jacquemin-Sablon
pQE30/PTB	from E. Tzima

Antibiotics

Ampicillin (amp)	Sigma
Tetracyclin (tet)	Roth
Kanamycin (kan)	Invitrogen

Antibodies

Anti eIF4B	Whatmann
Anti eIF4G	GE Healthcare
Anti eIF4A	Whatmann
Anti p110	prepared by Elena Tzima
Anti p170	gift from Shatsky lab
Anti His-tag	GE Healthcare
Anti PTB	prepared by Elena Tzima
Anti PCPB2	antibodies online
Anti unr	antibodies online
Anti La	antibodies online
Anti rabbit (secondary)	Whatmann
Anti mouse (secondary)	Whatmann

Bacterial Strains

E.coli XL1-blue
E.coli BL21
E.coli BL21 gold
E.coli BL21 plyss

Expression

ITAFs can be expressed recombinantly in *E.coli*. Since these proteins are RNA binding proteins, they can have toxic effects on the bacterial host when expressed in abundance. Bacteria therefore often store recombinant protein in insoluble inclusion bodies or modify the plasmid so the protein is not expressed at all. The expression and purification conditions had to be optimized for each protein.

PTB

pQE30 containing PTB was transformed into *E.coli* XL1-blue cells and a culture grown over night in LB medium containing 250 µg/ml ampicillin (amp) at 37 ℃. The overnight culture was

diluted 1:1'000 with LB medium containing amp and grown at 37 °C until the OD_{600} reached 0.6; then expression of PTB was induced with 100 mM IPTG. The expression continued for 3 hours at 37 °C before cells were harvested.

Cells were pelleted at 5'000 rpm for 15 min in a Beckmann centrifuge (rotor JA14) and resuspended with standard washing buffer, pelleted again and resuspended with standard lysis buffer. Cells were then lysed by sonication (2 min). Lysates were cleared by centrifugation for 20 min at 12'000 rpm in a Beckmann centrifuge (rotor JA20).

Lysates were directly transferred onto NiNTA beads equilibrated with standard lysis buffer and incubated at 4 °C for 30 min. Beads were then washed thrice with standard lysis buffer before PTB was eluted with standard lysis buffer containing 100 mM imidazol.

Quality and quantity of recombinant PTB was checked on SDS-PAGE.

PCBP2

This protocol is modified from Blyn et al. (1997). peT22b PCPB2 was transformed into *E.coli* BL21plyss cells and a culture grown over night in LB medium containing 250 µg/ ml amp at 37 °C. The overnight culture was diluted 1:1'000 with LB medium containing amp and grown at 37 °C until the OD_{600} reached 0.4; then expression of PCBP2 was induced with 100 mM IPTG. The expression continued for 4 hours at 28 °C before cells were harvested.

Cells were pelleted at 5'000 rpm for 15 min in a Beckmann centrifuge (rotor JA14), resuspended in PCBP2 lysis buffer. Cells were then lysed by sonication (2 min). Lysates were cleared by 20 min centrifugation at 12'000 rpm in a Beckmann centrifuge (rotor JA20).

Lysates were precipitated with 20 % ammonium sulfate at 4 °C, centrifuged at 5'000 rpm in a Beckmann centrifuge (rotor JA20) and pellets resuspended in PCPB2 lysis buffer. Then the solution was transferred onto equilibrated TALON beads and incubated at 4 °C for 30 min. Beads were transferred to a column and connected to a peristaltic pump. The column was washed thrice with PCBP2 lysis buffer before PCBP2 was eluted with PCBP2 elution buffer.

Quality and quantity of recombinant PCBP2 was checked on SDS-PAGE.

Unr

pRSET-unr was transformed into *E.coli* BL21 cells and a culture grown over night in LB medium containing 250 µg/ml amp at 37 °C. The overnight culture was diluted 1:1'000 with LB medium containing amp and grown at 37 °C until the OD_{600} reached 0.6; then cells were pelleted and medium removed. Cells were resuspended in LB medium containing amp and expression of unr was induced with 100 mM IPTG. The expression continued for 2 hours at 37 °C before cells were harvested.

Cells were pelleted at 5'000 rpm for 15 min in a Beckmann centrifuge (rotor JA14), resuspended in unr lysis buffer and frozen at -20 °C for 16 h. Cells were then lysed by sonication (5 min). Lysates were cleared by 20 min centrifugation at 12'000 rpm in a Beckmann centrifuge (rotor JA20) and the supernatant was discarded.

The cell debris containing inclusion bodies was resuspended in unr resuspension buffer containing 8.5 M urea at room temperature. The resuspension was incubated with NiNTA beads at room temperature for 12 h. Beads were then poured into a column and connected to a peristaltic pump that ran at 1.5 ml/min. The column was washed with unr-resuspension buffer containing 8.5 M urea for 15 min. Then the column was washed with unr- resuspension buffer containing only 6 M urea for 10 min, and then the column was washed with unr-resuspension buffer containing 4 M urea, then 3 M urea. After washing with resuspension buffer containing 3 M urea the column was transferred to a cold room and all subsequent steps were performed at 4 ℃. The column was washed with unr-resupension buffer containing 2 M, 1 M and 0 M urea for 10 min each. Then unr was eluted with unr elution buffer.

Quality and quantity of unr was checked on SDS-PAGE.

La

pLaGST contains 2 tags for purification, a C-terminal GST-tag and an N-terminal His-tag. pLaGST was transformed into *E.coli* BL21 cells and a culture grown over night in LB medium containing 50 µg/ml kanamycine (kan) at 37 ℃. The overnight culture was diluted 1:1'000 with LB medium containing 50 µg/ml kan and grown at 37 ℃ until the OD_{600} reached 0.8; then expression of La was induced with 100 mM IPTG. The expression continued for 4 hours at 37 ℃ before cells were harvested.

Cells were pelleted at 5'000 rpm for 15 min in a Beckmann centrifuge (rotor JA14) and resuspended with standard washing buffer, pelleted again and resuspended in standard lysis buffer. Cells were then lysed by sonication (2.5 min). Lysates were cleared by 20 min centrifugation at 12'000 rpm in a Beckmann centrifuge (rotor JA20).

Lysates were directly transferred onto equilibrated NiNTA beads and incubated at 4 ℃ for 30 min. Beads were transferred to a column and connected to a peristaltic pump. The column was washed thrice with standard lysis buffer before La was eluted with standard lysis buffer containing 100 mM imidazol.

Quality and quantity of recombinant La was checked on SDS-PAGE.

eIF4B, eIF4A and eIF4A R362Q

peT15b was transformed into *E.coli* BL21 cells and a culture grown over night in LB medium containing 250 µg/ml amp at 37 ℃. The overnight culture was diluted 1:1'000 with LB medium containing 250 µg/ml amp and grown at 37 ℃ until the OD_{600} reached 0.8; then expression was induced with 100 mM IPTG. The expression continued for 4 hours at 37 ℃ before cells were harvested.

Cells were pelleted at 5'000 rpm for 15 min in a Beckmann centrifuge (rotor JA14) and resuspended with standard washing buffer, pelleted again and resuspended in standard lysis buffer. Cells were then lysed by sonication (2 min). Lysates were cleared by 20 min centrifugation at 12'000 rpm in a Beckmann centrifuge (rotor JA20).

Lysates were directly transferred onto equilibrated NiNTA beads and incubated at 4 ℃ for 30 min. Beads were transferred to a column and connected to a peristaltic pump running at 1.5 ml/min. The column was washed thrice with standard lysis buffer before eIF4A or B was eluted with standard lysis buffer containing 100 mM imidazol.

Quality and quantity of recombinant protein was checked on SDS-PAGE.

eIF4G (p100)

peT15b was transformed into *E.coli* BL21 cells and a culture grown over night in LB medium containing 250 µg/ml amp at 37 ℃. The overnight culture was diluted 1:1'000 with LB medium containing 250 µg/ml amp and grown at 22 ℃ until the OD_{600} reached 0.5; then expression was induced with 100 mM IPTG. The expression continued for 4 hours at 22 ℃, before cells were harvested.

Cells were pelleted at 5'000 rpm for 15 min in a Beckmann centrifuge (rotor JA14) and resuspended with standard washing buffer, pelleted again and resuspended in standard lysis buffer. Cells were then lysed by sonication (2 min). Lysates were cleared by 20 min centrifugation at 12'000 rpm in a Beckmann centrifuge (rotor JA20).

Lysates were directly transferred onto equilibrated NiNTA beads and incubated at 4 ℃ for 30 min. Beads were transferred to a column and connected to a peristaltic pump running at 1.5 ml/min. The column was washed thrice with standard lysis buffer before p100 was eluted with standard lysis buffer containing 100 mM imidazol.

Quality and quantity of recombinant protein was checked on SDS-PAGE.

Transformation

Electroporation

Cells were grown in LB medium containing 15 µg/ml tetracyclin (tet) to an OD_{600} of 0.6. Then the medium was removed and cells were washed twice with cold ddH_2O. Cells were resuspended in cold ddH_2O and stored with 20 % Glycerol at -75 °C.

50 µl cells, 1-5 µl Plasmid and 50 µl ddH_2O were mixed in an electroporation cuvette and shocked with 2500 V for 10s. 800 µl LB medium was added to the cuvette, the mix transferred to an eppendorf tube and incubated at 37 °C for 1 h while shaking. Transformed cells were plated on LB-agar using the appropriate antibiotic.

Heat shock

Cells were grown in LB medium containing 15 µg/ml tetracyclin (tet) to an OD_{600} of 0.6. Then the medium was removed and cells were washed twice with cold 30 mM $CaCl_2$. Cells were resuspended in cold 30 mM $CaCl_2$ and stored with 20 % Glycerol at -75 °C.

50 µl cells and 1-5 µl Plasmid were incubated on ice for 20 min. The cells were shocked at 42 °C for 30 s, then cooled on ice for 2 min. 800 µl LB medium was added and the mixture was incubated at 37 °C for 1 h while shaking. Transform ed cells were plated on LB-Agar using the appropriate antibiotics.

SDS-PAGE

Acrylamide/bis-acrylamide (1:39) was mixed with separating gel buffer, 10 % APS and TEMED were added and a 12 % gel poured between two glass plates. After the separating gel polymerized, the 4 % stacking gel was prepared by mixing acrylamide/bis acrylamide (1:39) with stacking gel buffer, 10 % APS and TEMED. Samples were mixed with 4 x loading buffer and boiled at 95 °C for 5 min before loading to the gel. Gels were run at 80 V for 1 h or until the blue loading dye ran out.

Gels were then stained with coomassie stain for 30 min and destained over night in destaining solution.

Silverstaining of SDS-PA Gels

For very low amounts of protein gels were stained using the silver staining method developed by Samuel (1953).

After running of SDS-PA Gels, the gel was incubated in 50 % ethanol for 30 min.

Then the gel was washed 5 times 5 min in ddH_2O and incubated for 3 min in Silver stain solution (SSS) 1. After washing 4 times for 10 min with ddH_2O gels were incubated for 30 min in SSS 2 and subsequently washed 3 times with 2.5 % sodium carbonate.
Bands were visualized by incubation with SSS 3 until bands appear. Visualization reaction was stopped by addition of 10 % acetic acid after approximately 7 minutes.

Western blot

After SDS-PAGE proteins were transferred to nylon membranes via electroblotting.
Following SDS-PAGE the gel was washed in cathode buffer for approximately 5 min.

Blot sandwich was assembled in the following order:

Anode
3 layers of filter paper soaked in cathode buffer
Gel soaked in cathode buffer
Membrane wetted with 100 % methanol then soaked in anode buffer 2
Filter paper soaked in anode buffer 2
2 layers of filter paper soaked in anode buffer 1
Cathode

Proteins were transferred for 75 min at 143 mA.
The membrane was then blocked over night with 5 % skim milk powder or Rotiblock at 4 ℃.
After blocking the membranes were washed twice for 5 min with TBS-T, followed by incubation at room temperature for 1 h with primary antibody diluted in 5 % skim milk or Rotiblock. Dilutions of antibodies were specific for each antibody, ranging from 1:500 to 1:10’000.
After incubation the membrane was washed 4 times for 10 min with TBS-T and then incubated with the secondary antibody in 5 % skim milk or Rotiblock for 1 h at room temperature. The secondary antibodies were diluted 1:10’000.
Again membranes were washed for 4 times for 10 min with TBS-T before proteins were detected using the ECL detection kit on X-ray films.

Reconstitution of initiation complexes

Buffers

Gradient buffer

15 mM	Tris-HCl (pH 7.5)
0.5 mM	$MgCl_2$
80 mM	KCl
1 mM	DTT

Sucrose buffer

50 mM	Tris-HCl (pH 8.4)
6 mM	$MgCl_2$
60 mM	NaCl
10 mM	DTT

TBE-buffer

90 mM	Tris-Borat
2.5 mM	EDTA
0.625 %	Acetic acid

TAE-buffer

40 mM	Tris-HCl (pH 7.5)
40 mM	Na-acetate
2 mM	EDTA
2.5 %	Acetic acid

UV-crosslinking buffer

5 mM	Tris-HCl (pH 7.4)
5 mM	$MgCl_2$
5 mM	DTT
5 mM	rATP
5 mM	rGTP
25 %	Glycerol

Cell extracts

Rabbit reticulocyte lysate	Promega
	Green Hectars
	prepared by Shatsky laboratory
S30 ascites cell extract	prepared by Shatsky laboratory
HeLa extract	Cilbiotech

Plasmids

PGL3 containing

- PV IRES
- EMCV IRES
- β-actin 5’ UTR

Primers

Forward primer:

T7GL4	adds T7 promoter upstream of IRES, anneals to vector sequence cgccgtaatacgactcactatagggagcttatcgataccgtcg 3’	5’

Reverse primers:

Flsh	binds at nt 100 in the firefly luciferase coding region 5’ attgttccaggaaccagggcg 3‘
Flsh+A	binds at nt 100 in the firefly luciferase coding region, adds 50 thymines to the end of the template resulting in a polyA tail on the RNA 5’ttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttattgttccaggaaccagggcg 3’
Actinshort	binds at nt 200 in the firefly luciferase coding region 5’ ggaacaacacttaaaatcgcagtatccggaatg 3’
Actinshort+A	binds at nt 200 in the firefly luciferase coding region, adds 50 thymines to the end of the template resulting in a polyA tail on the RNA 5’ttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttggaacaacacttaaaatcgcagtatccggaatg3’

Preparation of templates for transcription

DNA template was prepared from PGL3 plasmids. Primers were designed to add a promoter for the T7 RNA polymerase to the 5’ end of the template.

PCR reaction:

100 ng	Template (plasmid)
0.25 mM	dNTPs
1 x	Taq buffer
2.5 mM	$MgCl_2$
1 pmol	forward Primer
1 pmol	reverse Primer
3 u	Taq DNA-Polymerase

Cycling program:

95 ℃ for 3 min

80 ℃ for 1 min

70 ℃ for 1 min

64 ℃ for 1 min

72 ℃ for 3 min

95 ℃ for 45 s

80 ℃ for 5 s

75 ℃ for 5 s — 33 cycles

67 ℃ for 1 min

72 ℃ for 1.5 min

72 ℃ for 5 min

4 ℃

Transcription with hot UTPs

For capped RNA a 10 µl reaction was prepared using:

1 µg	Template
1 x	Capping mix
0.5 mM	m^7GTPG
1 x	transcription buffer
2 µl	^{32}P labelled UTPs
20 u	T7 RNA polymerase

The reaction was incubated at 37 ℃ for 1.5 h.

For uncapped RNA a 10 µl reaction was prepared using:

1 µg	Template
0.25 mM	ATP, GTP, CTP mix or rNTPs
1 x	transcription buffer containing $MgCl_2$
0.1 mM	UTP
2 µl	^{32}P labelled UTPs
20 u	T7 RNA polymerase

The reaction was incubated at 37 ℃ for 1 h.

If RNA was used to fish proteins 2 nmol of biotinylated UTP was added to this reaction.

Lithiumchloride precipitation

RNA was mixed with 1 volume of 4 M LiCl and incubated on ice for 1.5 h. After precipitation the tube was centrifuged at 4 ℃ for 15 min at 13.2 00 rpm in a benchtop centrifuge. The pellet was washed with 70 % Ethanol and air dried for 30 min at room temperature. The pellet was resolved in 1 volume of ddH_2O or nuclease-free water.

Sucrose Gradients

For stepwise gradients sucrose solutions from 10 % to 35 % (in 5 % increments) were prepared and the gradients were poured in 2 ml steps. Balanced gradients were frozen at -20 ℃ over night and slowly thawed in ice water before loading.

For linear gradients sucrose solutions from 5 % and 20 % were prepared in A-100 and 11 ml gradients poured using a gradient mixer. Linear gradients were used after cooling for 30 min at 4 ℃.

One 50 µl reaction of

24 µl	RRL
6 µl	HeLa extract
1 mM	m^7GTP
1 x	gradient buffer
10 mM	GMP-PNP

was pipetted and incubated at 30 ℃ for 5 minutes. Radioactively labelled RNA was added and the reaction was incubated for an additional 10 min. Then the reactions were diluted 1:3 using A-100 and loaded on the prepared gradients.
Initiation complexes were separated by centrifugation for 5.5 h at 40’000 rpm (rotor SW28). After centrifugation gradients were fractionated into 500 µl fractions. Each fraction was mixed with 2 ml Rotiszint and radioactivity measured in a Scintillation-counter.
When gradient fractions were needed for further protein analysis, radioactivity was counted in a Cherenkov counter, which allows fractions to be used for further experiments, since no scintillation enhancer has to be added.
Measured values were normalised to total radioactivity per reaction.

RelE-assay

RelE is a bacterial toxin that cleaves mRNA only in the P-site of ribosomes, which is an easy way to determine on which AUG codon translation initiated when ribosomes are stalled before subunit joining.

Radioactively labeled RNA was used in a translation reaction. The reaction was stopped at the stage of 48S complexes by addition of GMP-PNP and complexes isolated via sucrose gradients. RNA from identified 48S complex is then digested by the bacterial toxin RelE. 1 µl (ca. 10 u) RelE is used to digest RNA in 100 µl of pooled fractions at 30 ℃ for 30 min.

After digestion the RNA was cleaned with phenol-chloroform and precipitated in the presence of 0.25 mM rNTPs with 100 % Ethanol.

Digested RNA as well as a control undigested RNA was then separated on a PA gel and bands were visualized on X-ray film or using a phosphoimager. Cleavage patterns of digested and undigested RNA were compared to a marker.

Binding of biotinylated RNA to streptavidin beads

Fractions derived from sucrose gradients or total RNA protein complexes from translation reactions were loaded on a streptavidin-Sepharose column.

Streptavidin-sepharose beads were centrifuge at maximum 3'000 rpm in an Eppendorff benchtop centrifuge.

The column was equilibrated with A-100 through extensive washing. Biotinylated RNA was allowed to bind for 1 hour at 4 ℃ while shaking.

Beads were then washed twice with A-100 (+ 10mM Mg-acetate) and proteins were eluted with 3 µl of RNase A/RNase T1 mix in 60 µl of A-100. A second elution with 10 mM biotin in A-100 was performed when necessary. Eluted proteins were checked on SDS-PAGE.

Depletion of HeLa extract

Magnetic streptavidin-coupled beads were equilibrated with A-100 by extensive washing. Beads were incubated with an equal amount of HeLa extract for 15 min on ice to block unspecific binding sides. Excess HeLa extract was removed and beads were washed with A-100 thrice.

To deplete 30 µl of HeLa extract 3 µg biotinylated RNA and 30 µl of HeLa extract were mixed with 15 µl of blocked beads and incubated on ice while shaking.

After incubation the depleted extract was removed and placed on ice while the beads were washed 3 times with A-100. Depleted extracts and beads were treated with micrococcal nuclease. 2.5 mM $CaCl_2$ and 3 units of micrococcal nuclease were mixed with 40 µl of

depleted extract or beads in A-100 and incubated at room temperature. The nuclease digestion was stopped by adding 0.25 mM EGTA, which chelates the Ca^{2+} ions essential for micrococcal nuclease to work.

UV-crosslinking

Radioactively labeled RNA was mixed with cell extract (RRL or HeLa) or pure proteins in crosslinking buffer and incubated at 30 ℃ for 10 m in to allow protein binding to RNA. The mix was irradiated with UV light (260 nm and 320 nm) for 30 min on ice, irreversibly crosslinking RNA to proteins. After irradiation the mix was digested with RNase A for 1 h at 37 ℃ and proteins separated on SDS-PAGE. Proteins were fixed in the gel by incubating in destaining solution for 5 min and subsequently dried on filter paper. X-ray film was exposed to dried gels for 1-3 days at -70 ℃ to visualize bands.

Translation of reporter constructs

Buffers

Hybridization buffer	
2 %	DIG blocking reagent
5x	SSC
0.035 %	Laurolylsarcosine
0,02 %	SDS
50 %	Formamide

20 x SSC	
3 M	NaCl
0.3 M	$Na_3C_6H_5O_7$

Maleic acid buffer	
100 mM	Maleic acid
150 mM	NaCl

TAE-buffer	
40 mM	Tris-HCl
40 mM	Na-acetate
2 mM	EDTA
2.5 %	acetic acid

TBE-buffer	
90 mM	Tris-Borat
2.5 mM	EDTA
0.625 %	Acetic acid

Passive lysis buffer	
1 ml	5x passive lysis buffer
4 ml	PBS

Firefly luciferase buffer	
25 mM	Gly-Gly
15 mM	$MgSO_4$
0.1 mM	EDTA
33.3 mM	DTT
1 mM	rATP

PBS	
137 mM	NaCl
2,7 mM	KCl
4.3 mM	Na_2HPO_4
1.5 mM	KH_2PO_4

Firefly luciferase substrate	
470 µM	Luciferin
20 mM	Tricine
1,07 mM	$(MgCO_3)_4Mg(OH)_2$
2,67 mM	$MgSO_4$
0.1 mM	EDTA
33.3 mM	DTT
270 µM	Coenzyme A
530 µM	rATP

10 x capping mix
0.5 mM rGTP
5 mM rATP
5 mM rCTP
5 mM rUTP

PA-Gel	
7 M	urea
15 %	acrylamide/bis-acrylamide

FA-buffer	
50 mM	EDTA
80 %	Formamide
0.1 %	Bromphenol blue
0.1 %	Xylenecyanol

Plasmids

pCITE PV1 VP1-2A	from T. Skern
pD5	from C. Jünemann
pGL3 containing - Polio IRES + Luciferase - EMCV IRES + Luciferase - β-globin 5' UTR + Luciferase - β-actin 5' UTR + Luciferase	from I. Shatsky
pMPolio	from E. Tzima

Oligonucleotides

Primers

Mutation of miRNA-326mat binding site in PV IRES

326mutIRESfwd	5’ctcaacgggcgagtgtagcttaggctg 3‘
Luciferase1400rev	5’gccacacccttaggtaaccca 3‘
PVIRESfwd	5‘gatttaggtgacactatagaatagatcttaaaac 3‘
326mutIRESrev	5‘cagcctaagctacactcgcccgttgag 3‘

Silent mutation of miRNA-326* binding site in firefly luciferase

326mutLucifwd	5’catagaacggcttgcgtcaga 3‘
Luciferase1400rev	5’gccacacccttaggtaaccca 3‘
PVIRESfwd	5‘gatttaggtgacactatagaatagatcttaaaac 3‘
326mutLucirev	5‘tctgacgcatgccgttctatg3‘

Templates for transcription

T7GL4	5’cgccgtaatacgactcactatagggagcttatcgataccgtcg 3‘
End of firefly	5’ ttaacttgtttattgcagcttataatgg 3’
End of firefly +A	5’ ttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttaacttgtttattgcagcttataatgg 3’

miRNAs

miRNA-127mat	5’ ucggauccgucugagcuuggcu 3’	5'Phosphate
miRNA-127*	5’ ccugcugaagcucagagggcucugauu 3’	5'Phosphate
miRNA-326mat	5’ ccucugggcccuuccuccag 3’	5'Phosphate
miRNA-326*	5’ ggaggcagggccuuugugaaggcg 3’	5'Phosphate
miRNA-422amat	5’ acuggacuuagggucagaaggc 3’	5'Phosphate
miRNA-422a*	5’ ucucugucccugagccaagc 3’	5'Phosphate
miRNA-326matmutcomp	5’ ccucgccccccuuccuccag 3’	5’Phosphate
miRNA-326*mutcomp	5’ ggaggcagggggggugggaaggcg 3’	5’Phosphate

Probes

		5'DIG
U6 snRNA	caggggccatgctaatcttctctgtatcg	3'DIG
		5'DIG
326	ctggaggaagggcccagagg	3'DIG
		5'DIG
422a	ggccttctgaccctaagtccag	3'DIG
		5'DIG
127	agccaagctcagacggatccga	3'DIG

Cell lines

HeLa	
SH-SY5Y	gift from A. Brehm

Lysates

Rabbit Reticulocyte Lysate	Promega
HeLa- extract	Cilbiotech

Preparation of templates for in vitro transcription

<u>By digest</u>

DNA template was prepared by cutting the pMPolio or pD5 plasmid with SmaI 700 bp downstream of the luciferase gene and purifying the linearized plasmid from a 1 % agarose-gel followed by phenol-chloroform extraction.

<u>By PCR</u>

DNA template was prepared from the pCITE or PGL3 containing the PV IRES plasmids using PCR. Primers were designed to add a promoter for the T7 RNA polymerase to the 5' end of the template.

PCR reaction:

100 ng	Template (plasmid)
0.25 mM	dNTPs
1 x	Taq buffer
2.5 mM	$MgCl_2$

1 pmol	forward Primer
1 pmol	reverse Primer
5 u	Taq Polymerase

Cycling program:

95 ℃ for 3 min	
80 ℃ for 1 min	
70 ℃ for 1 min	
64 ℃ for 1 min	
72 ℃ for 3 min	
95 ℃ for 45 s	
80 ℃ for 5 s	
75 ℃ for 5 s	33 cycles
67 ℃ for 1 min	
72 ℃ for 1.5 min	
72 ℃ for 5 min	
4 ℃	

PCR products were cleaned using phenol-chloroform extraction.

Phenol-Chloroform extraction

DNA was mixed with 1 volume of phenol, centrifuged for 2 min and the aqueous upper phase transferred to a new Eppendorff tube. It was then mixed with 1 volume of a 1:1 phenol:chloroform mix, centrifuged for 2 min and the aqueous upper phase transferred to a new eppendorff tube. 1 volume of chloroform was added, mixed, centrifuged for 2 min and the aqueous upper phase transferred to a new eppendorff tube.

To this 1/10 volume of 3 M Na-acetate and 3 volumes of 100 % ethanol were added. Then the DNA was precipitated over night at -20 ℃, foll owed by 30 min centrifugation at 13'000 rpm. The pellet was washed twice with 500 µl of 70 % ethanol and air dried at room temperature for 30 min, before being solved in 50 µl of ddH_2O.

Preparation of miRNA duplexes

Guide strand and passenger strand RNAs were purchased from biomers.net as single stranded RNA oligonucleotides. Guide strands are annotated with the suffix "mat", and passenger strands are annotated with an asterisk " * ". Equimolar amounts of the two strands

were hybridized by rapid heating to 80 °C in a water bath and slow cooling to room temperature. Double stranded RNAs were stored at -20 °C and used for 2-3 weeks.

In vitro transcription

For capped RNA a 10 µl reaction was prepared using:

1 µg	Template (pD5 derived)
1 x	Capping mix
0.5 mM	m^7GTPG
1 x	Transcription buffer
20 u	T7 RNA polymerase

The reaction was incubated at 37 °C for 1.5 h.

For uncapped RNA a 10 µl reaction was prepared using:

1 µg	Template (PV IRES, EMCV IRES, β-actin 5' UTR, pD5)
0.25 mM	rNTPs
1 x	transcription buffer containing $MgCl_2$
20 u	T7 RNA polymerase

The reaction was incubated at 37 °C for 1 h.

Quality and quantity of the resulting RNA was controlled on 1 % agarose-gels.

In vitro translation

In vitro transcripted mRNA was mixed with RRL. The KCl concentration was adjusted to 135 mM (Kozak 1989) by adding 2 M KCl, taking into account that the endogenous KCl concentration is approximately 113 mM K^+ and IRESs are more efficient under high salt conditions. The reaction was incubated at 30 °C for 45 min and luciferase activity measured.
For miRNA experiments single stranded (ss) miRNA was serially diluted to 400 ng/µl, 200 ng/µl, 100 ng/µl and 50 ng/µl and 2 µl of each dilution added to the reaction.
All reactions were performed in duplicates.

Tissue culture

Cells were grown in DMEM with Penicillin/Streptamycine and 10 % fetal bovine serum (FBS) at 37 °C with 5 % CO_2. Cultures were passaged in 25 cm^2 flasks. For transfections cells were plated on 24-well-plates, for northern blots cells were grown in 75 cm^2 flasks.

Transfection

All transfections were performed in 24-well-plates in duplicate.

Cells were transiently transfected using Lipofectamine 2000™ with a luciferase construct (IRES, capped or uncapped) mRNA and different amounts of miRNA.

500 µl cells were plated into 24-well-plates with antibiotic-free DMEM, containing 10 % FBS and grown over night to approximately 80 % confluency. 50 µl of plain DMEM were mixed with 2 µl of Lipofectamine and incubated for 5 min at room temperature. mRNA and miRNA duplexes were mixed in 50 µl of plain DMEM, the two mixes were combined and incubated at room temperature for 20 min. Medium on the cells was changed to plain DMEM and the transfection mix was added. After 4 h of incubation at 37 °C with 5 % CO_2 cell viability was tested with the WST-1 assay. Subsequently cells were lysed using 150 µl of 1 x passive lysis buffer and luciferase activity was determined.

WST-1 Test

The WST-1 test measures the activity of enzymes from the respiratory chain and can therefore by used as a reliable measure of the overall cell viability in the well.

The WST1 reagent was diluted with colorless DMEM 1:50. Growth medium was removed from the cells, and the cells were washed with PBS, before 200 µl of WST1/DMEM was added. After 30 min incubation with WST1, 100 µl of medium was transferred to a 96-well plate and color intensity was determined in a microplate reader at 450 nm.

Luciferase reporter assay

Luciferin was prepared in luciferase buffer and stored at -20 °C. Cell lysates were cleared by centrifugation at 4'000 rpm for 5 min at 4 °C. Luciferase activity was measured using a Berthold luminometer with automatic injection of luciferin. 100 µl of luciferin solution was added to the sample and luminescence measured for 20 s, the measurement was repeated and both values were averaged.

Isolation of total small RNA from cells

Cells were grown to 90 % confluency in 75 cm^2 flasks. Medium was removed, cells were washed with PBS and subsequently lysed with lysis buffer supplied by the miRNA isolation kit from Roche. miRNAs were separated from long RNAs by precipitating mRNAs with ethanol, before binding to column. Not precipitated miRNAs were found in the flowthrough, were subsequently precipitated and bound to a new column, where they were eluted with ddH_2O.
The exact buffer composition is not disclosed since it is patented by Roche. I followed the instructions in the manual of the kit.

PAGE

15 % PA-Gels were prepared as described for SDS-PAGE and RNA samples mixed with FA-buffer. Samples were loaded on gel and run at 20 mA until the blue dye band reached the middle (~20 min) of the gel if separating small RNAs and until the dye ran out (~1 h) for long mRNAs.

Northern Blot

Blotting sandwich was assembled in the following order:

Anode
3 layers of filter paper soaked in TBE
Membrane soaked in TBE
PA-Gel
3 layers of filter paper soaked in TBE
Cathode

After separation on PA-Gels RNAs were transferred to nylon membranes via semi-dry electroblotting at 150 mA for 55 min. RNA was crosslinked to the membrane by exposure to UV-light (260 nm) for 7 min. The membrane was then incubated at 68 ℃ with hybridization buffer for 1 h. During the incubation 10 µl of the appropriate DIG-labeled probe was boiled at 95 ℃ for 1 min, then cooled down on ice for 5 min before being mixed with 25 ml of hybridization buffer. The probe-mix was added to the membrane and incubated at 68 ℃ over night (at least 12 hours).
The membrane was then washed twice for 20 min with 2 x SSC containing 0.1 % SDS at 68 ℃, followed by washing it twice for 20 min with 0.5 x SSC containing 0.1 % SDS at 68 ℃.

The membrane was blocked for 30 min at room temperature with 1.5 % skim milk powder in maleic acid and subsequently incubated for 1 h with ANTI-DIG antibodies in a dilution of 1:10'000 in 1.5 % skim milk powder in maleic acid.
The membrane wash washed thrice with 0.3 % tween in maleic acid buffer for 10 min before being incubated with DIG detection solution for 1 min. An X-ray film was applied to the membrane and exposed over night (10 - 24 h).

Introduction of mutations into the pMPolio construct

Two sites were identified for mutation, a miRNA-326mat binding site in the PV IRES and a miRNA-326* binding site in the firefly luciferase. Both mutations were achieved using the same approach.
Two PCR reactions were performed using Pfu-DNA-Polymerase, starting or ending at the point of mutation (mutation primers). Designed mutation primers introduced the mutated sequence at the desired site, unchanged primers (outer primers) were used as forward and reverse primer respectively. The two PCR products from the first round were then used as template for a second round of PCR using the outer primers.

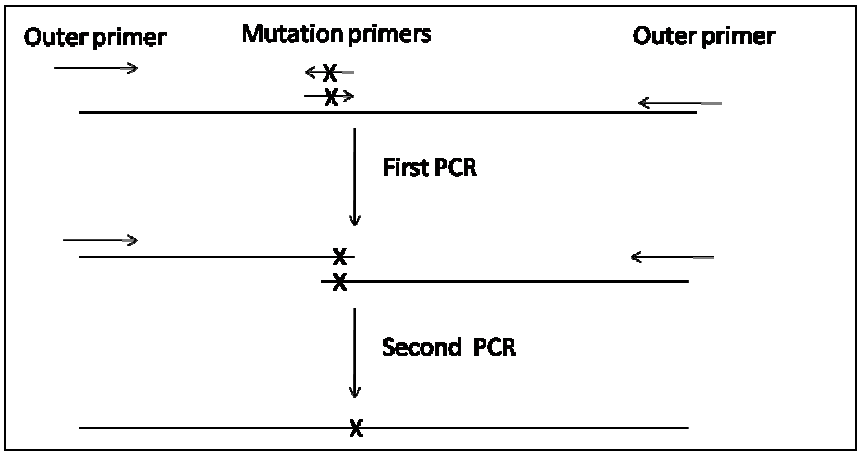

Figure 1: Schematic description of the mutation strategy used to mutate miRNA-326 binding sites in the PV IRES and firefly luciferase.

The outer primers were chosen to include cutting sites for restriction endonucleases into the product. The product of the second round of PCR and the pMPolio plasmid were cut with those enzymes, then purified from 1 % agarose gels and the PCR product was ligated into the vector using T4-Ligase.

Plasmids were transformed into *E.coli* XL1-blue and plated on LB plates containing amp. Ten colonies were picked, grown in LB-medium containing amp, subsequently plasmids were prepared and send for sequencing with GATC company.

Online tools

General:

http://www.ebi.ac.uk/clustalW/

http://www.basic.northwestern.edu/biotools/oligocalc.htm

miRNAs:

http://www.vita.mbc.nctu.edu.tw

http://www.**microrna**.org

http://www.targetscan.org

RNA/protein interaction:

http://mfold.bioinfo.rpi.edu

http://www.matrixscience.com

Equipment

ÄKTA FPLC System	GE Healthcare
Benchtop centrifuge	Eppendorff
Blotting membrane	Whatmann
Centrifuge	Beckmann/Sorvall
Clean Bench	Heraeus
CO_2-Incubator	Binder
Developing equipment for films	Agfa
Electric kettle	Ciatronic
Electroporator	Bioline
Gel-documentation	Biorad
Gel running apparatus	von Keutz
Geldryer	Beckmann
Heating block	Kleinfeld
Hybridiser	UVP
Incubator	Certomat
Luminometer	Berthold
Magnetic stirrer	Bender & Hobein
Micropipettes	Gilson

Microplate reader	BioTek
Microscope	Leica
Peristaltic pump	Gilson
Photometer	Eppendorff
Power supply	Pharmacia
Semi-dry blotter	Biorad
Shaker	Heidolph/CAT
Sonicator	Biorad
Scintillationcounter	Packard
Thermocycler	Eppendorff/Biometra
Ultracentrifuge	Beckmann
UV lamp	Packard
Rotors	Beckmann
SW27	
SW40	
Ti70	
Vortex	Bender & Hobein
Waterbath	Julabo

Chemicals

All chemicals were purchased from Roth unless stated otherwise.

Acrylamide/bis-acrylamide	
Agarose	
Ammonium sulfate	
Anisomycine	
Amoniumpersulfate	
Biotin	Roche
Calciumchloride	
Chloroform	Sarstedt
Dulbecco’s modified Eagle medium	
Dithiothreitol	
Ethylenediaminetetraacetic acid	
Ethanol	
Fetal bovine serum	
Formaline	

GMP-PNP	Sigma
Imidazol	
IPTG	
Lithium chloride	
Lipofectamine 2000™	Invitrogene
m^7GTP	Jena Bioscience
Magnesium acetate	
Magnesium chloride	
Medium Lucia Bertani	
Methanol	
Phenol	
Potassium chloride	
Rotiblock	
Rotiszint ™	
Sodium dedocylsulfate	
Skim milk powder	
Sodium acetate	
Sodium carbonate	
Sucrose	
Tetramethylethyldiamine	
Tris	
Tween 20	Sigma

Enzymes

EcoRI	New England Biolabs (NEB)
Pfu DNA polymerase	NEB
RelE	gift from I. Shatsky
RNase A	Fermentas
RNase T1	Sigma
SmaI	Fermentas
SP6 RNA Polymerase	Fermentas
T4 Ligase	NEB
T7 RNA polymerase	Fermentas
Taq DNA Polymerase	Promega
XbaI	Fermentas

Consumables

24-well plates	Sarstedt
25 cm^2 flasks	Greiner
75 cm^2 flasks	Greiner
96-well plates	Sarstedt
Blotting membrane	Whatmann
Eppendorff tubes	Sarstedt
Falcon tubes	BD Falcon
LB agar plates	Virology
Parafilm	American National Can
Pipettes	BD Falcon
Streptavidin coupled Sepharose beads	GE Healthcare
Streptavidin coupled magnetic beads	Invitrogen
Tips for micropipettes	Sarstedt
Vivaspin concentrator columns (cut off 30'000 Da)	Sartorius
X-ray film	Kodak

Kits

Miniprep KIT	Promega
Maxiprep	Macherey Nagel
Gel purification	GE Healthcare
miRNA isolation	Roche
DIG detection	GE Healthcare
ECL detection	Whatman/Promega

Results

Purification of eukaryotic initiation factors

Using the method established by Prof. Shatsky

Eukaryotic initiation factors (eIFs), especially eIF3 and eIF4F, are large multi-subunit proteins, which are post-translationally modified (Raught et al. 2000) and assembled by partially unknown processes (Fraser et al. 2004). Therefore it is nearly impossible to prepare active proteins recombinantly in *E.coli*. Consequently native eIF3 and eIF4F were purified from rabbit reticulocyte lysate (RRL).

Reticulocyte lysate is prepared by first injecting rabbits with phenylhydrazine hydrochloride which renders them anemic by destroying erythrocytes. This leads to an increased formation of erythrocyte progenitor cells, the reticulocytes (Borsook et al. 1952, Schreier & Staehelin 1973). This procedure was performed by Green Hectares (Wisconsin, USA), the only company worldwide that supplies large amounts of untreated RRL directly to researchers.

Erythrocytes and their progenitors, reticulocytes, are major places of protein synthesis; their main function is biosynthesis of hemoglobin, and they are therefore very suitable for purification of translation-related factors, such as ribosomes and eukaryotic initiation factors.

There are several methods of purification of initiation factors described in the literature (e.g. Benne et al. 1976, 1977, 1978, Merrick 1979, see table 1).

Article	**Factor**	**Steps**	**Methods**	**Amount of starting material**
Merrick et al. 1975	eIF2	7	ASP, ion-exchanger, size exclusion	600 rabbits
Thomas et al. 1979	eIF2	5	Heparin sepharose, ASP, size exclusion, ion-exchanger	4 l RRL
Thomas et al. 1979	eIF3	5	ASP, ion-exchanger, sucrose gradient, Heparin-sepharose	4 l RRL
Benne et al. 1976	eIF3	5	ASP, ion-exchanger, sucrose and glycerol gradients	90 rabbits
Pestova et al. 1996	eIF3	5	ASP, ion-exchanger, sucrose gradient	4 l RRL
Benne et al. 1978	eIF3	5	ASP, ion-exchanger, sucrose and glycerol gradients	120 rabbits
Schreier, Erni, Staehelin 1977	eIF4B	6	ASP, ion-exchanger, sucrose gradient	270 ml RSW
Thomas et al. 1979	eIF4B	5	Heparin-sepharose, ASP, sucrose gradient, ion-exchanger	4 l RRL

Pestova et al. 1996	eIF4F	5	ASP, ion-exchanger, sucrose gradient, cap-sepharose	4 l RRL

Table 1: Different purification methods for eukaryotic initiation factors described in the literature, ASP: ammonium sulfate precipitation, RSW: ribosomal salt wash, RRL: rabbit reticulocyte lysate

After several unsuccessful attempts, which will be described later, I purified factors using the protocol established in the laboratory of Prof. Shatsky at the Belozersky Institute for Physio-chemical Biology at the Moscow State University in Russia (e.g. Pestova et al. 1996). This protocol is based on work by Schreier & Staehelin (1973 and 1977) and Benne & Hershey (1976). Purification of initiation factors using Prof. Shatsky's protocol is described in the methods section and an overview is given in figure 1; results are presented here.

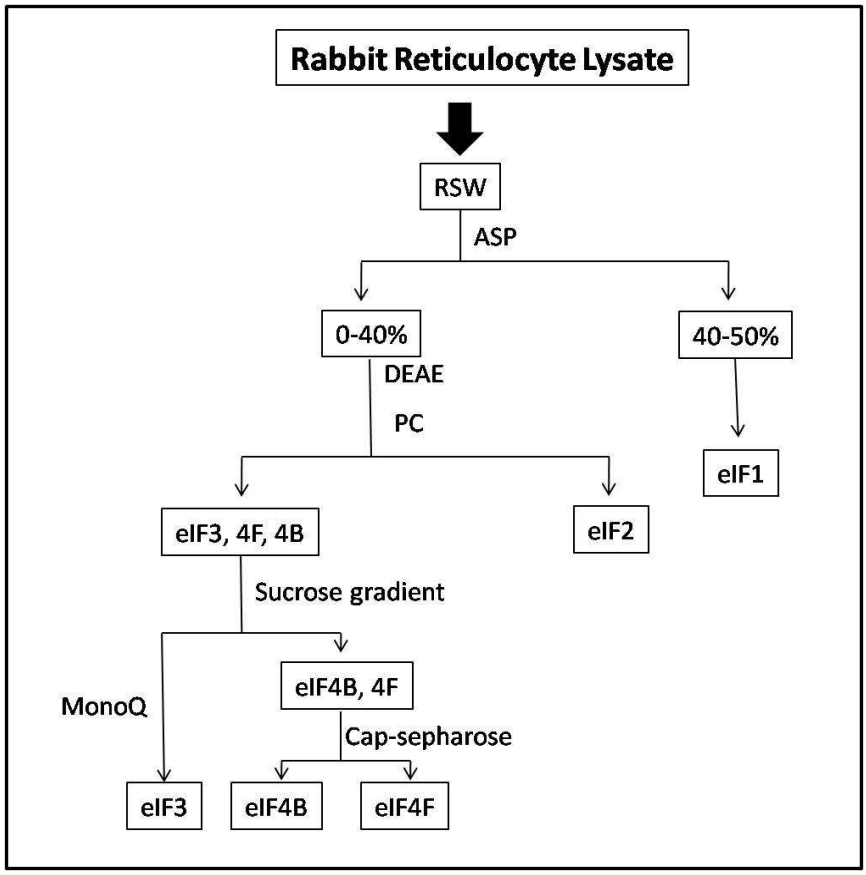

Figure 1: Schematic description of the method used to purify eukaryotic initiation factors 3, 4B and 4F from RRL. Factors eIF1 and eIF2 may be purified using this method but have not been purified here. Instead ASP fraction and phosphocellulose fractions were frozen and stored at -80℃.
RSW: ribosomal salt wash, ASP: ammonium sulfate precipitation, PC: phosphocellulose, eIF: eukaryotic initiation factor

Large amounts (4 liters) of RRL were obtained from the company Green Hectares and ribosomal salt wash (RSW) was prepared reducing the volume to about 600 ml.

Proteins in the RSW were differentially precipitated with ammonium sulfate (0 - 40 %, 40 - 50 %, 50 - 70 %). The 40 % ammonium sulfate fraction was subjected to ion-exchange chromatography, first on an anion exchanger, DEAE cellulose (Figure 2). Peak-fractions eluted from the DEAE cellulose column containing initiation factors were then loaded on a cation exchanger, phosphocellulose (PC) (Figure 3).

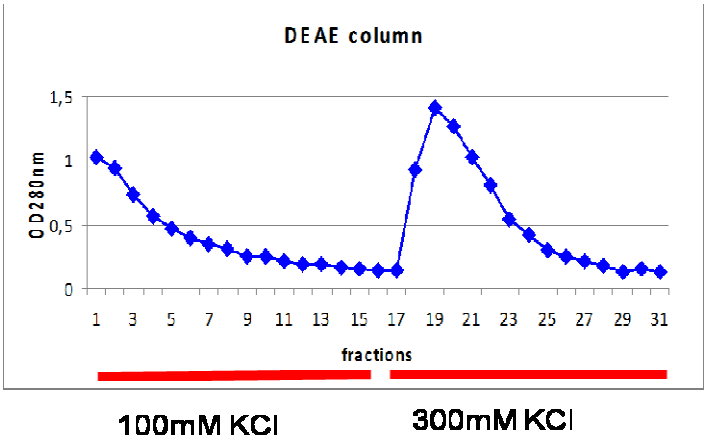

Figure 2: Third step of purification of eukaryotic initiation factors. 40 % ammonium sulfate precipitates of RSW were subjected to a DEAE sepharose column. 8 ml fractions were collected. Elution with A-300 (300 mM KCl) started at fraction 16, elution fractions (18 – 22) were pooled.

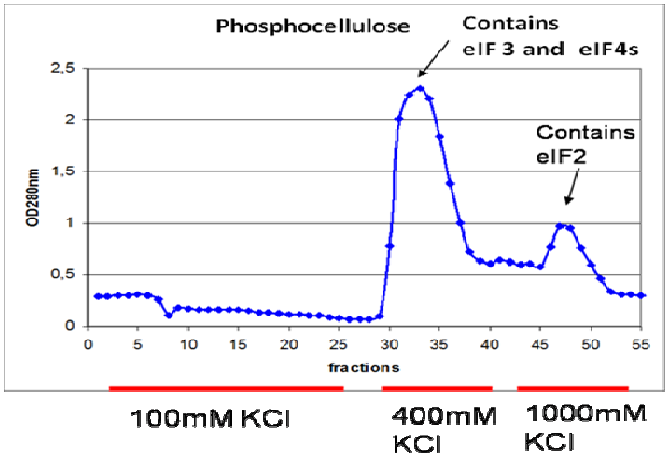

Figure 3: Fourth step of purification. Elution fractions from DEAE column were separated on a PC-column. Fractions of 8 ml were collected. Elution with A-400 (400 mM KCl) started at fraction 26, elution fractions containing eIF3 and eIF4F and B (30 - 35) were pooled. Elution with A-1000 (1 M KCl) started at fraction 43; eIF2 may be purified from elution with A-1000 but it is not relevant for the purification described here.

After the ion-exchange steps the amount of total protein in the final elution was only about a third of the 40 % ammonium sulfate precipitate (200 mg, compared with a 600 mg in the 40 % ammonium sulfate precipitate). The bulk of proteins from RRL (mainly hemoglobin and lipoxygenase (Borsook et al. 1952)) was removed after these steps.

Initiation factors were now almost pure. Consequently eIF3 and factors of the eIF4 family could be separated on 5 - 20 % sucrose gradients. Since the volume of the preparation was still rather high (7 ml), three or four gradients were run simultaneously. In figure 4 the reproducibility of sucrose gradients is shown.

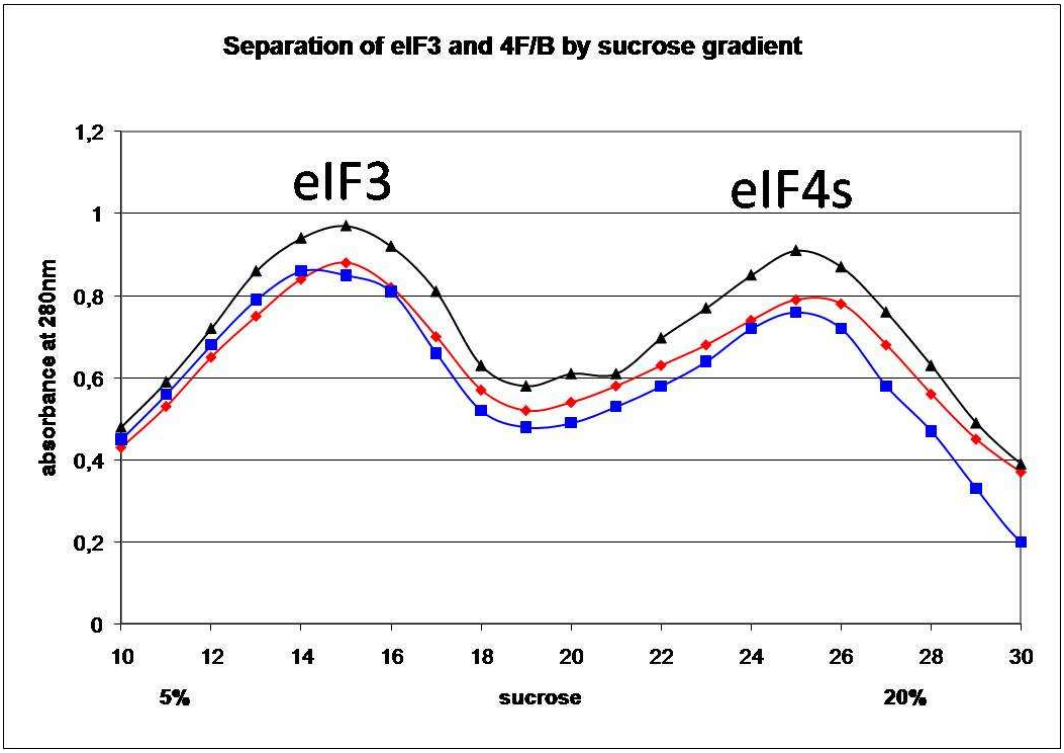

Figure 4: Three different sucrose gradients (5 - 20 %) were run to separate proteins eluted from the PC-column (figure 3). Sucrose gradients separate eIF3 (first peak) from eIF4s (second peak). A total of 200 mg of protein was loaded and 500 µl fractions obtained.

Pure eIF4F could be isolated from the second (eIF4) peak using Cap-Sepharose (Figure 5) as described in the methods section.

Unfortunately eIF4G was degraded in this preparation, resulting in purification of only the cap binding moiety eIF4E on the Cap-Sepharose, while eIF4A can be identified in the flow through.

Another preparation performed in Prof. Shatsky's laboratory however led to the recovery of complete eIF4F.

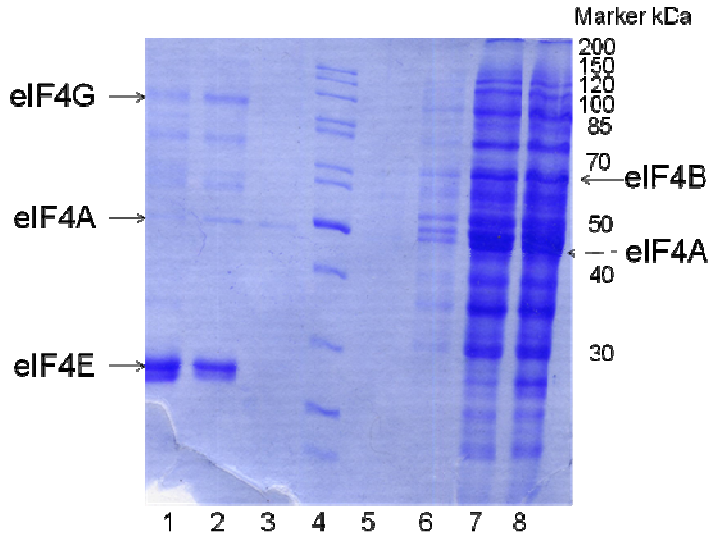

Figure 5: Separation of fractions from purification of eIF4F via Cap-Sepharose on a 12 % SDS-PAGE Gel stained with coomassie:

1 and 2: Elutions from column using m^7GTP

3, 5 and 6: Washes of column with A-100

4: Marker

7: Flow through containing eIF4A and eIF4B

8: Sample loaded on the column containing eIF4A, 4B and 4E

Pure eIF3 could be isolated from the first peak of the sucrose gradients (figure 4), unfortunately p170, the largest subunit of eIF3 was partially degraded as well (Figure 6 lanes 5 and 6). In fractions containing low amounts of eIF3, p170 seems to be at least partially intact, even though the concentration compared to p110 is very low, indicating that only few molecules of complete eIF3 were present. In intact eIF3 p110 and p170 are present in equimolar amounts (Hinnebusch 2006).

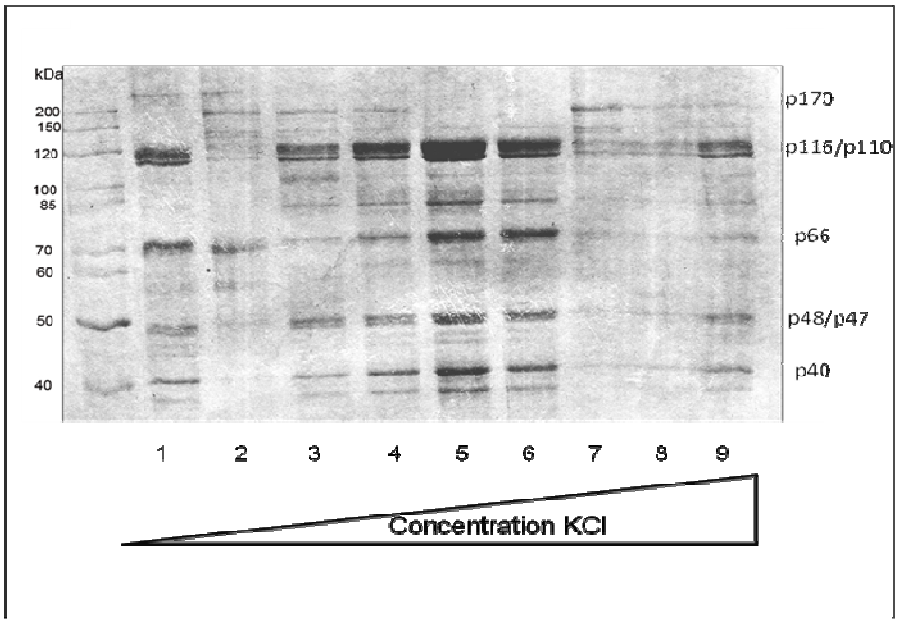

Figure 6: Purified eIF3 without subunit p170 in lanes 5 and 6. Fractions from purification of the eIF3 peak of sucrose gradients on a MonoQ column, eluted with a linear gradient from 0 to 500 mM KCl (lane 1 (0 mM) to lane 9 (500 mM KCl)) were separated on a 12 % SDS-Gel and the gel stained with coomassie. Prominent subunits of eIF3 are marked on the right.

All the degradation observed during this purification led me to check for protein integrity in the original RRL.

I found by western blot that p170 (figure 7) as well as eIF4G was already partially degraded in the starting material. Several degradation products can be seen at lower molecular weights, the strong band at ~72 kDa however seems to be unspecific binding of the antibody raised against p170.

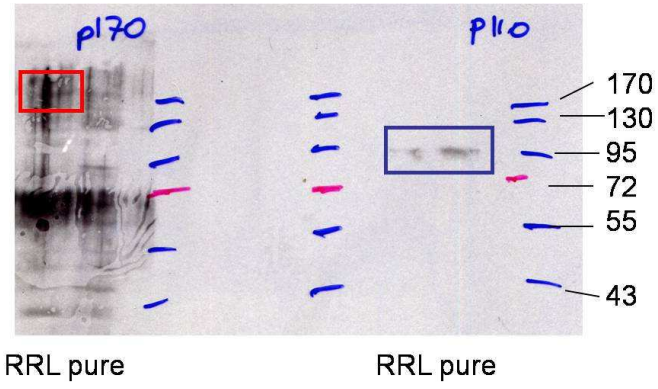

Figure 7: Western blot using primary antibodies against p170 (left) and p110 (right) subunits of eIF3 in complete RRL (RRL) and purified eIF3 (pure) from the same preparation of RRL. Secondary antibodies against mouse IgG were coupled with alkaline phosphatase for detection.

Degradation of p170 in RRL seems to be a rather common problem when purifying factors (I. Shatsky), therefore purification from a different source, like HeLa or Krebs cells would be preferable. This however proved impossible since it is very difficult to acquire the necessary amounts of cell extracts.

Purification of initiation factors using Phenyl Sepharose

Several methods of purification of native eukaryotic translation initiation factors from diverse sources have been published (summarized in table 1). All of the methods described up to date need to employ at least five purification steps to result in reasonably pure eIF3 and eIF4F. All of them use a combination of separation by charge (ion-exchangers) and size (gradients, size exclusion columns). Even though separation by thiolgroups (Safer et al. 1976) and hypoxyapatite, which is a modification of ion-exchange chromatography, have been tried, they did not proof effective enough to become part of an established protocol.

I tried a new approach, separating proteins by the hydrophobicity of their side chains on a Phenyl Sepharose column. In principle this method is similar to precipitation with ammonium sulfate, a step that is included in almost all established purification protocols (e.g. Benne et al. 1976, 1977, 1978, see table 1).

Fractionation by ammonium sulfate precipitation however is rather imprecise since only large increments (0 - 40 %, 40 - 50 %, 50 - 70 %) can be used, resulting in eIF3 and proteins from the eIF4 family precipitating together in the 0 - 40 % ammonium sulfate fraction. Instead of using ammonium sulfate I used a Phenyl Sepharose column. Hydrophobic side chains of amino acids form complexes around the phenyl moiety of the column, essentially precipitating at the column. Decreasing the salt content (starting from 1.7 M ammonium sulfate) of the buffer then elutes the proteins by changing their hydrophobicity due to decreased ionic strength of the buffer, releasing them from the phenyl-group. The salt concentration can be controlled rather tightly using an ÄKTA FPLC machine with computer-controlled buffer mixer, therefore it should be possible to elute initiation factors at their perfect salt conditions, resulting in reasonably pure factors after just one step of purification.

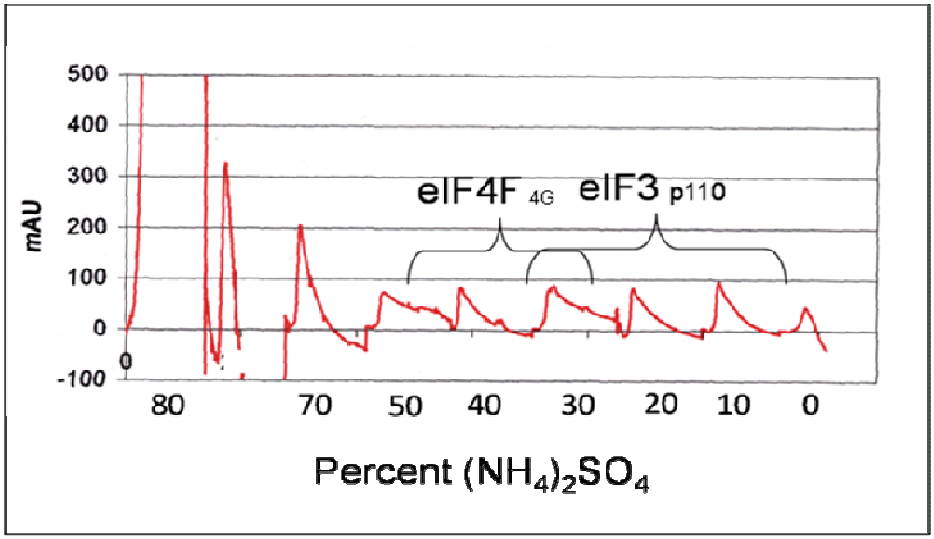

Figure 8: Purification of initiation factors using Phenyl Sepharose. Proteins were loaded to the column at 1.7 M ammonium sulfate ($(NH_4)_2SO_4$). The percentage of ammonium sulfate in the elution buffer was lowered in 10 % increments by adding an identical buffer without ammonium sulfate resulting in proteins being separated by their hydrophobicity. Protein content of fractions was measured in relative absorbance units (mAU) at 320 and 280 nm. Initiations factors were identified in the fractions using western blot detecting with antibodies against part of eIF4F, eIF4G, and a subunit of eIF3, p110.

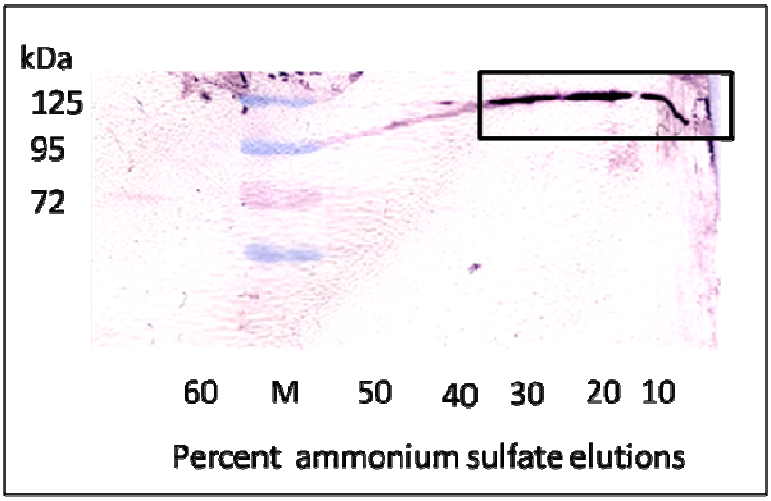

Figure 9: Western blot to identify eIF3 subunit p110 in elution fraction from Phenyl Sepharose column as described in figure 8. Detected bands are marked with a black square. Fractions were separated on 12 % SDS-PAGE gels and transferred to a phosphocellulose membrane, primary antibody was raised against p110 in mouse and detected using a secondary anti-mouse IgG antibody coupled to alkaline phosphatase.

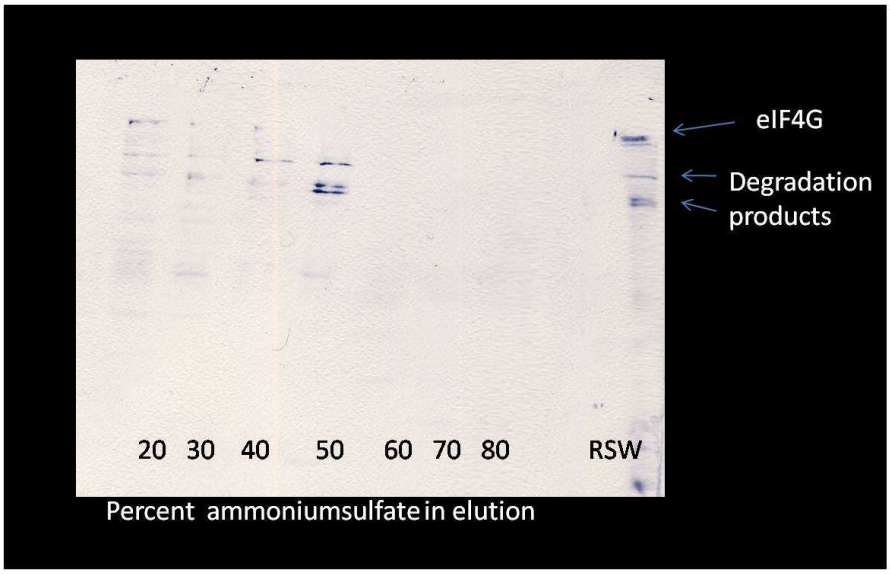

Figure 10: Western blot to identify eIF4F subunit 4G in elution fraction from Phenyl Sepharose column as described in figure 8. eIF4G and degradation products can be detected. Fractions were separated on 12 % SDS-PAGE gels and transferred to a phosphocellulose membrane by semi-dry electroblotting. Primary antibody was raised against eIF4G in rabbit and detected using a secondary anti rabbit IgG antibody coupled to alkaline phosphatase.

However, this attempt on purification was not successful, since initiation factors could be detected over several fractions (Figure 8, 9 and 10) indicating that they do not have a distinct perfect salt condition. This is not surprising taking into account, that eIF3 and eIF4F are large multi-subunit proteins with different local charges and hydrophobicity. This result shows a similar salt preference of factors as the established ammonium sulfate precipitation, where eIF3 and eIF4F precipitate between 0 and 40 % of ammonium sulfate, however it suggests that a higher increment of ammonium sulfate (up to 45 % or 50 %) may be better to include all of the eIF4F present in RSW.

If using hydrophobic interaction chromatography additional steps of ion-exchange and maybe size exclusion are necessary to gain pure factors. This method is therefore no improvement over established methods using simple ammonium sulfate precipitation.

To reach the aforementioned conclusion many experiments were necessary, since one major problem was that I started initial experiments with a very low amount of RRL (5 - 10 ml) or RSW (1.2 - 3 ml), when compared to established protocols (Table 1) resulting in no peaks above background level and not enough material eluted to be detected on SDS-PAGE or even western blot. After noticing this problem I attempted purification (shown in figure 8) with RSW from 1.2 liters of RRL resulting in factors only just detectable by western blot and not

seen as distinct bands on coomassie SDS-PAGE. 1.2 l of RRL is still at the low end of the amounts used in previously published protocols, so the low yield is not surprising. It is expected that about 67 % of factors present in RRL can be isolated to purity (Safer et al. 1975) the rest is lost in void volumes and on columns. Therefore it is crucial to start with enough material, that 67 % of initiation factors are still enough to be detectable.
In order not to waste more expensive RRL I decided to take advantage of an already well established protocol and the expertise offered by Prof. Shatsky as described above.

Purification of ITAFs

IRES trans-acting factors (ITAFs) are proteins normally not involved in translation, but in other parts of the RNA metabolism, like splicing or shuttling. ITAFs are per definition RNA binding proteins. Most of them do not bind sequence-specific, but bind to RNA in general or on common motifs, for example PABP binds to the polyA tail. Expression of some of them in *E.coli* was therefore challenging, since they prove toxic to the cells and were expressed only in small amounts (PCBP2, La) or sequestered into inclusion bodies (unr). For each protein a separate expression and purification protocol had to be established (see Materials and Methods). Variations in growth temperature, OD_{600} at time of induction, concentration of IPTG and different strains of *E.coli* were used to optimize expression. However unr had to be purified from inclusion bodies, while PCBP2 could only be bound to Ni-NTA beads when an additional step of ammonium sulfate precipitation was used to concentrate proteins before incubation with beads. Finally all ITAFs were purified and tested on SDS-PAGE gels.

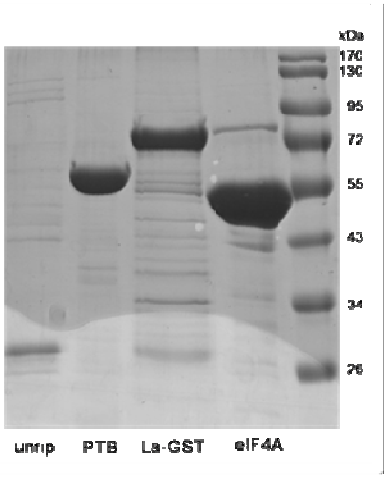

Figure 11: Three known ITAFs, unrip, PTB and La-GST, as well as the initiation factor eIF4A were expressed recombinantly and purified using His-Tag affinity chromatography. Elutions from beads were separated on a 12 % SDS-PAGE gel and stained with coomassie.

Conclusion

All factors necessary for future experiments, reconstitution and UV crosslinks could be obtained in high quality and sufficient quantities. This took longer than expected due to an unsuccessful attempted to establish a new purification protocol for initiation factors. Recombinant expression of initiation factors and ITAFs was optimized and purification protocols established.

How to reconstitute the poliovirus IRES?

Reconstitution of an mRNA means, in this case, initiation of translation up to the stage of 48S-initiation complexes or 80S ribosomes without formation of peptide bonds and elongation. Formed ribosomal complexes are visualized by toe-printing or sucrose gradient centrifugation.

The reconstitution is, under ideal conditions, performed using only purified factors as done with the EMCV (Pestova et al. 1996) and HCV IRES (Pestova et al. 1998) and capped beta-globin mRNA (Dimitriev et al. 2003). Use of purified components (initiation factors, ribosomes, t-RNA) allows to establish the minimal factor requirement of an IRES element to initiate translation.

Reconstitution of the PV IRES from purified components has not been successful up to now. As a type 1 IRES, PV IRES has a large requirement for trans-acting factors, which are present in HeLa extract but not in RRL (Dorner et al. 1984). All ITAFs identified for the PV IRES can be prepared recombinantly or purified from cell extracts and were used in reconstitution assays. A combination of all known ITAFs with all eIFs, t-RNA and purified ribosomes did not lead to a functional reconstitution of the PV IRES from pure components. The PV IRES can be reconstituted however using RRL supplemented with HeLa extract (e.g. Figure 12), indicating the presence of one or more additional ITAF(s) in HeLa extract. Before another attempt on reconstitution with purified components can be undertaken the missing ITAF(s) have to be identified. I tried to find those components using two different approaches, first isolation of proteins bound to the PV IRES in 48S complexes after sucrose gradient centrifugation and second depletion of HeLa cell extract with the PV IRES bound to magnetic beads.

Isolation of proteins from 48S complexes

ITAFs and initiation factors bound to the PV IRES for translation initiation are present in the 48S initiation complex when bound to the starting codon AUG before subunit joining. To be able to purify proteins from 48S initiation complexes translation reactions were stopped by

GMP-PNP (Hinton et al. 2007) which inhibits the function of eIF2 and therefore subunit joining and dissociation of factors. The initiation process is halted at the stage of 48S complexes and these complexes are accumulated. The complexes can then be separated on sucrose gradients (5 - 20 % sucrose). Visualization of complexes in sucrose gradient fractions was achieved by labeling RNA with radioactive UTPs. After fractionation of the gradient the amount of radioactivity in each fraction can be used to identify 48S complexes.

Using biotinylated PV-IRES mRNA all proteins bound to the mRNA can be linked to a Streptavidin column via the biotin-tags on the mRNA and can then be eluted by cleaving the mRNA with RNases. mRNA in 48S complexes was selected via the gradient profiles and subsequently used in binding assays. To distinguish between mRNA present in 48S complexes and mRNA with bound proteins just present in those fractions and to certainly identify complexes in the gradient, a dominant negative mutant of eIF4A R362Q (Pause et al. 1994) was added to a control reaction. This mutant reversibly inhibits any translation initiation dependent on eIF4A by competing with the wild type form, leading to gradient profiles without 48S complexes (figure 12).

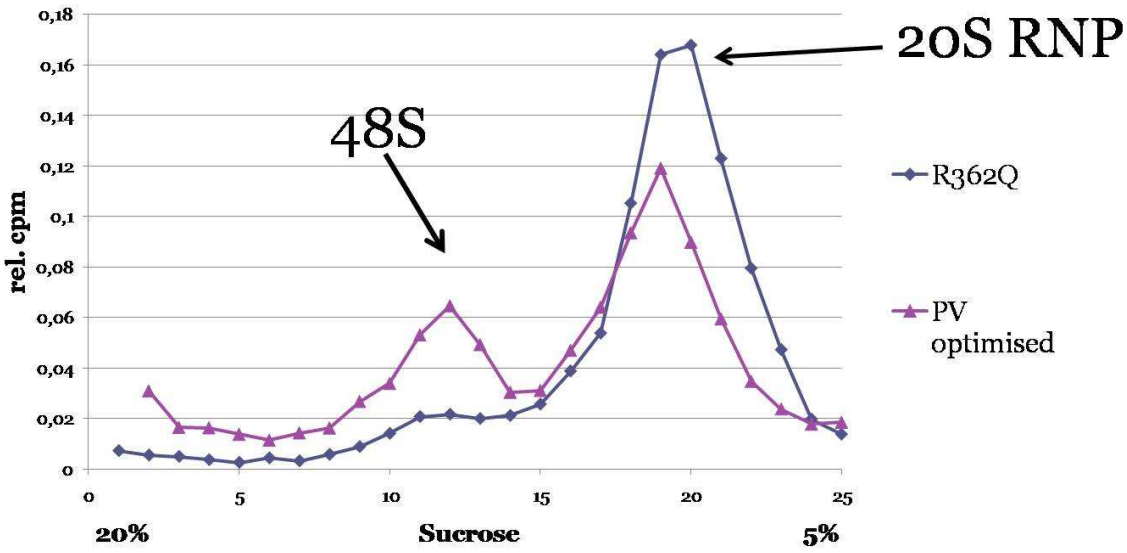

Figure 12: 5-20 % sucrose gradient separating 48S complexes in an optimized translation reaction on the PV IRES in RRL supplemented with 20 % Hela extract inhibited (diamonds) and not inhibited (triangles) by the dominant negative mutant of eIF4A, R362Q. 48S complexes are present in fractions 11-14, the relative counts per minutes (rel.cpm) were normalized on total radioactivity in the gradient.

Optimization of translation initiation

The first step was to optimize the translation reaction for maximum yield of initiation complexes.

As a template for initiation I used short in vitro transcribed RNAs in which the reporter firefly luciferase was only partly (100 nt) present after the PV IRES. This enables a good separation of 48S complexes from mRNPs (Hinton et al. 2007). mRNA was labeled with two differently modified UTPs, α-^{32}P radioactive UTPs and biotinylated UTPs. Radioactive UTPs were necessary to visualize gradient profiles. The biotin-tag was added to purify proteins in a later step.

Since the goal was to “fish” proteins using biotin-tags, internally biotinylated mRNAs were employed for optimization. No significant difference in formation of 48S complexes could be found between biotinylated and non-biotinylated RNA indicating that the effect of biotin on secondary structure of the IRES and formation of 48S complexes is negligible (Figure 13) at the used concentration of biotinUTP in the RNA.

When translation reaction, inhibited with GMP-PNP, are separated on 5 - 20 % sucrose gradients, two forms of RNA-protein complexes can be observed, 48S initiation complexes (at middle sucrose-concentration) and 20S mRNP complexes (at low sucrose-concentrations). In most cases the mRNP complexes are much more pronounced than 48S complexes.

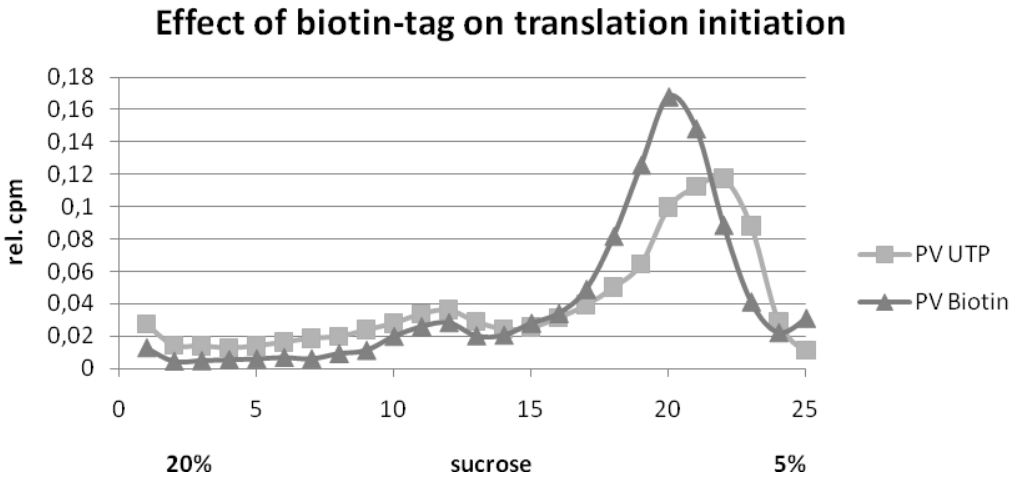

Figure 13: 5 - 20 % sucrose gradient separating 48S complexes in a not yet optimized translation reaction with RRL supplemented with 20 % Hela extract using biotinylated (triangles) and not biotinylated (diamonds) PV IRES mRNA. 48S complexes are present in fractions 11-14. The relative counts per minutes (rel.cpm) in each fraction were normalized on total radioactivity in the gradient.

Consistent with previous studies (Brown & Ehrenfeld 1979, Dorner et al.1984) I found that addition of 20 % HeLa extract vastly increased translation efficiency in RRL (figure 14), since several known ITAFs like La and unr are missing from RRL, but are present in HeLa extract. HeLa extract itself is not able to support translation, most likely due to high amounts of RNases present in the extract (figure 14 and 15).

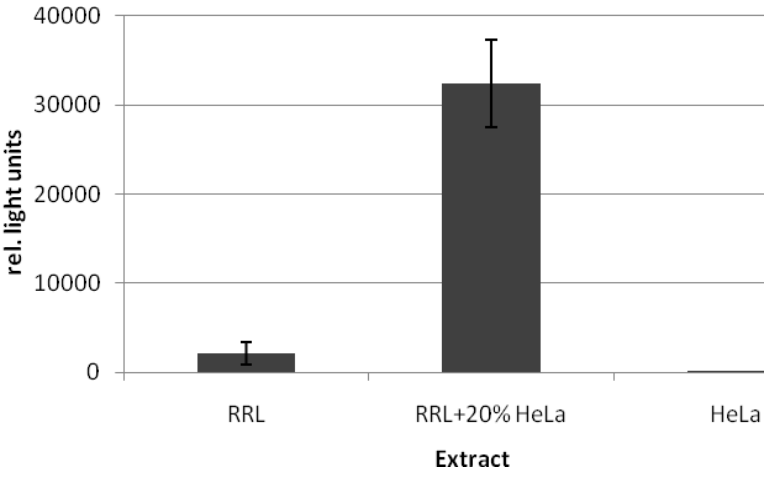

Figure 14: Translation efficiency at the PV IRES in RRL, RRL supplemented with 20 % of HeLa extract and pure HeLa extract; measured as relative light units produced by firefly luciferase activity from an in vitro translation reaction using a firefly luciferase reporter under the control of the PV IRES. The experiment was performed twice in triplicate.

The effect of HeLa extract on the formation of 48S complexes was additionally tested by sucrose gradient to exclude an effect of HeLa extract on other stages of translation. Here the short RNA without the full length luciferase reporter ORFs was used and translation inhibited at the 48S stage using GMP-PNP. As seen in figure 15 almost no initiation complexes were formed when using not supplemented RRL or pure HeLa extract. When using pure HeLa extract most radioactivity and therefore RNA was detected in the very light fractions of the gradient, indicating that not even RNA-protein complexes without ribosomal subunits were formed. This is most likely due to degradation of RNA in HeLa extract by endogenous RNases.

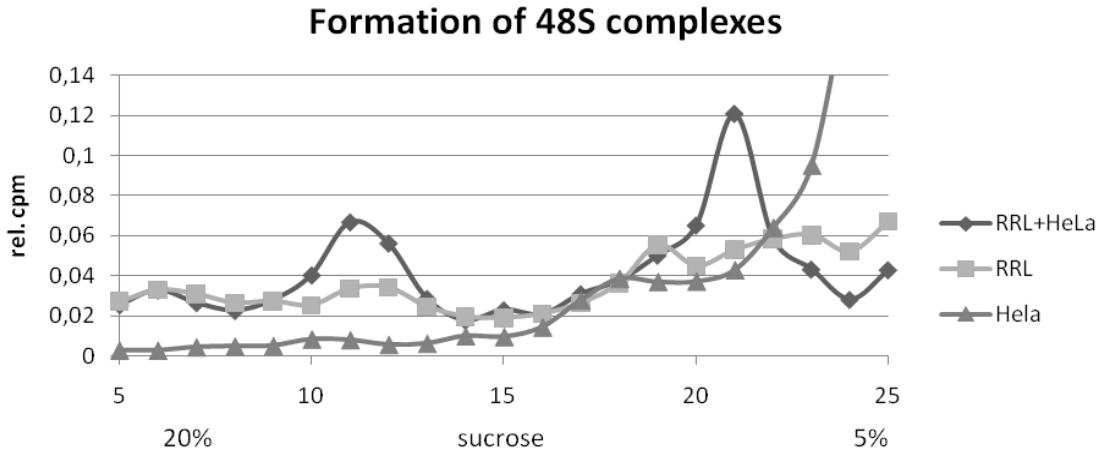

Figure 15: Formation of 48S complexes in RRL (squares), RRL supplemented with 20 % HeLa extract (diamonds) and HeLa extract (triangles). Initiation reaction was stalled by addition of GMP-PNP and 48S complexes were enriched. Reaction mixture was separated on 5 % to 20 % sucrose gradients, 48S complexes can be seen at fractions 11-13. The relative counts per minutes per fraction (rel. cpm) were normalized to total radioactivity in the gradient.

Commercially available RRL is treated with micrococcal nuclease to eliminate endogenous mRNAs which would compete with reporter constructs for factors, ribosomes and amino acids (Pelham & Jackson 1976). But even nuclease treated RRL still contains capped mRNAs or fragments thereof, which compete with the IRES for initiation factors and ribosomal subunits. These mRNAs are mostly globin mRNAs, which are very stable and protected from RNases by the globin stability complex (Weiss & Liebhaber 1995) and therefore hard to completely eliminate by RNase digestion without also eliminating ribosomal RNAs. Addition of m^7GTP, which inhibits cap-dependent, but not IRES dependent translation by inhibiting binding of eIF4E to the cap, allows the IRES to prevail in this competition for ribosomes and factors (Svitkin et al. 1996, Bi & Goss 2000, figure 16).

Additionally, it is known that polyA tails stimulate translation of mRNAs (Gallie 1991). Since PV naturally has a polyA tail (Semler et al. 1984), addition of 50 As to the 3' end of the construct increased translation efficiency (figure 16) as well as the yield of 48S complexes in the gradients (Bergamini et al. 2000, Paulous et al. 2003, figure 17 and 18 on the next page).

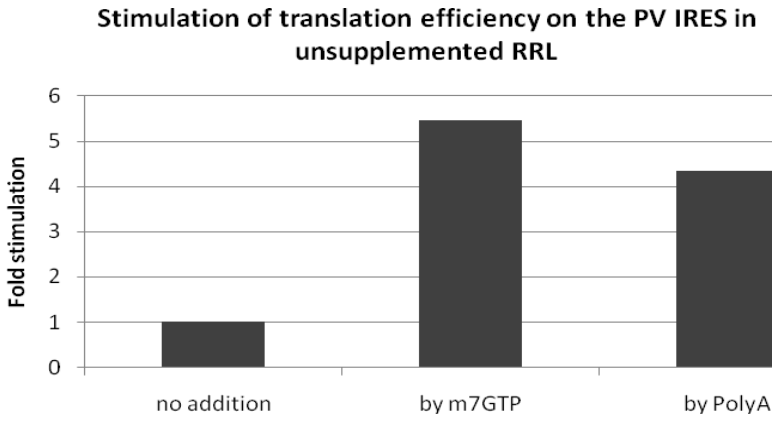

Figure 16: Addition of m^7GTP and adding of 50As to the end of the mRNA controlled by the PV IRES stimulated translation efficiency of a firefly luciferase reporter construct in not supplemented RRL five and four fold respectively. Experiments were performed twice in duplicate.

PolyA did not stimulate formation of 48S complexes at the EMCV IRES (Figure 18). Even though several cases of stimulation of translation on the EMCV IRES by polyA tails have been described (Hruby & Roberts 1977, Bergamini et al. 2000) in the literature, this could not be repeated here.

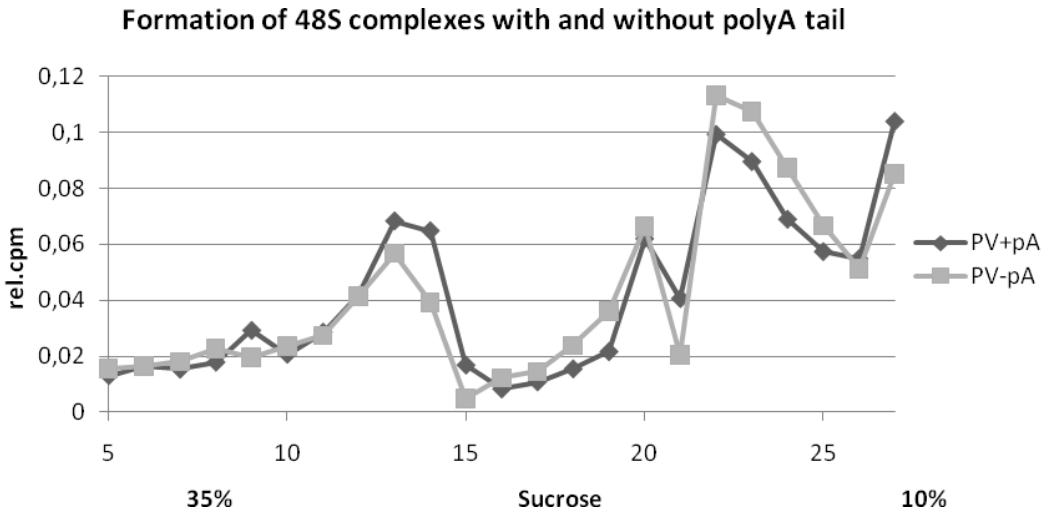

Figure 17: Effect of a $polyA_{50}$ tail on formation of 48S complexes. Translation initiation was performed using short in vitro transcribed mRNAs with (diamonds) or without (squares) addition of 50 As at the 3' end. Translation was inhibited at the stage of 48S initiation complexes by addition of GMP-PNP and complexes separated on 10 - 35 % sucrose gradients.

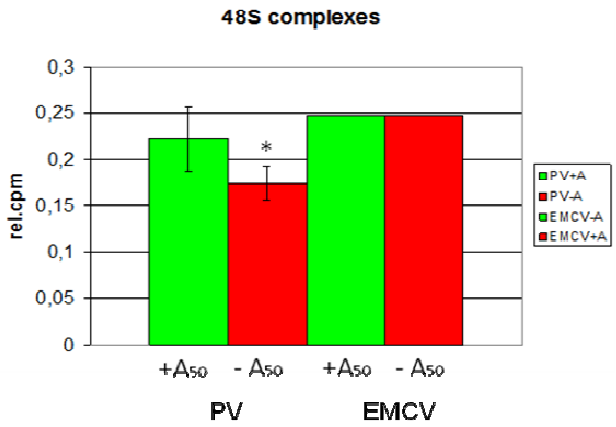

Figure 18: Summary of five gradients separating 48S complexes assembled at the PV IRES (performed as described in figure 17) and two gradients separating 48S complexes assembled at the EMCV IRES. A significant stimulation of PV IRES dependent translation by polyA_{50} tail can be seen, significance was tested using ANOVA p=0.02.

After optimizing the translation conditions the optimal amount of RNA in one reaction needed to be elucidated; different amounts of mRNA were tested (figure 19) and an optimal amount of 0.5 µg RNA in a 50 µl reaction was chosen as a compromise between low yield at higher RNA concentration (purple line) versus low amount of total RNA (red line) being able to bind to Streptavidin beads.

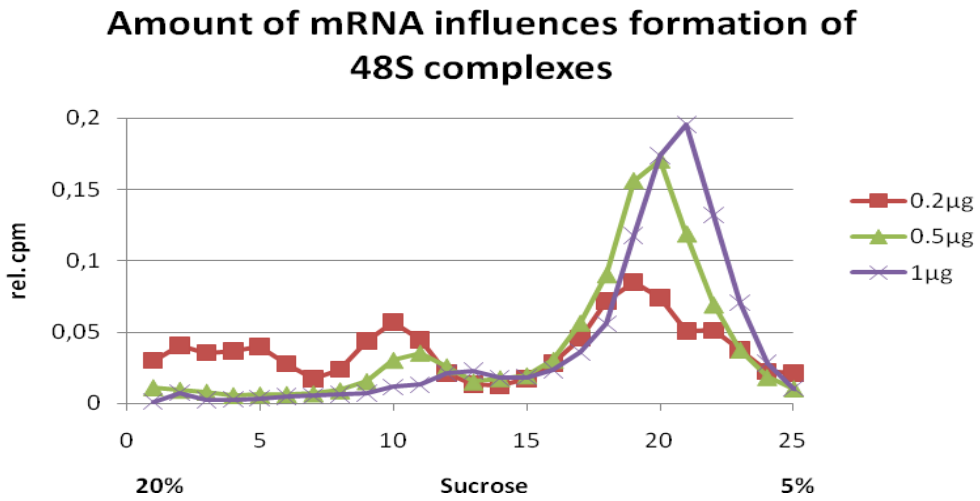

Figure 19: Formation of 48S complexes on the PV IRES in RRL supplemented with 20 % HeLa extract with 0.2 µg (red), 0.5 µg (green) and 1 µg (purple) of PV IRES RNA added to 50 µl of translation reaction. Translation reactions were stopped by addition of GMP-PNP and separated on 5 % to 20 % sucrose gradients.

Using 0.5 µg of mRNA addition of p100, a fragment of eIF4G, which is produced in infected cells by the viral protease 2A, lead to an increased formation of 48S complexes (as described in Ohlmann et al. 1995, Borman et al. 1997, Roberts et al. 1998, figure 20).

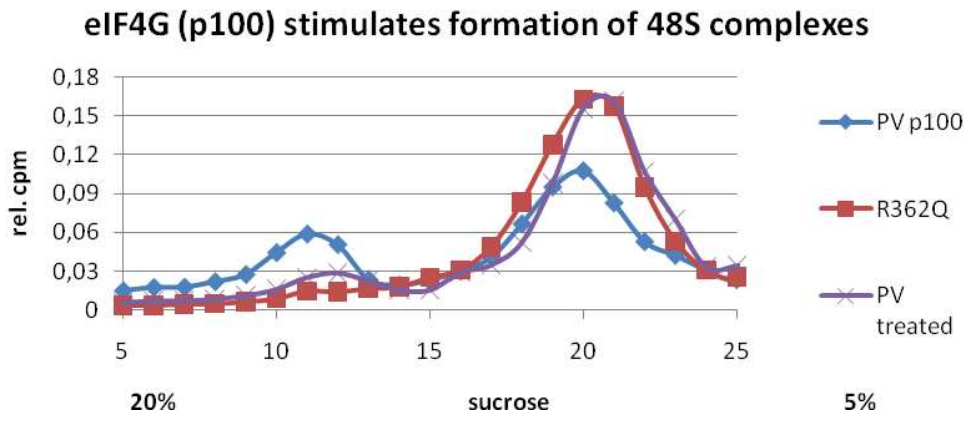

Figure 20: Formation of 48S complexes in RRL supplemented with 20 % HeLa extract with (diamonds) or without (crosses) addition of recombinant eIF4G (p100). As a control, translation reaction was inhibited using the dominant negative mutant of eIF4A R362Q (squares). Initiation was stopped by addition of GMP-PNP and translation reactions separated on 5 % to 20 % sucrose gradients.

Reconstituting the PV IRES from a polyadenylated construct in RRL supplemented with 20 % HeLa extract, addition of m^7GTP and eIF4G (p100), yielded an amount of 48S complexes much larger than described previously (Ochs et al. 1999, 2002). The amount of proteins (initiation factors and ITAFs) in the 48S peak was therefore thought sufficient to isolate unknown proteins that are part of the 48S initiation complex with biotinylated mRNA.
To further increase the amount of protein obtained in the 48S peak, peak fractions from three gradients run simultaneously were combined or the reaction was up-scaled to the volume limits of the centrifugation tube (300 µl).

Optimisation of binding to beads

48S complexes from sucrose gradients were bound to Streptavidin Sepharose beads via the internal biotin-tags in the PV-RNA and proteins eluted from the beads using RNase A, RNase T1 or a mix of both RNases. Digestion of the RNA releasing only proteins from the beads that are bound to RNA even though parts of the RNA remained bound. Proteins binding

unspecifically to Sepharose beads or biotin are retained on the beads. Eluted proteins were then separated by SDS-PAGE and stained with the very sensitive silver staining method.
As a first step to optimize elution several RNases were tested using radioactive EMCV RNA bound directly to beads (figure 21). After elution radioactivity in the eluate was measured and compared to radioactivity still bound to the beads. A mix of the two most active RNases A and T1 was used in experiments. However performance of RNases on naked RNA does not necessarily reflect performance when proteins are bound to the RNA masking potential cleavage sites.

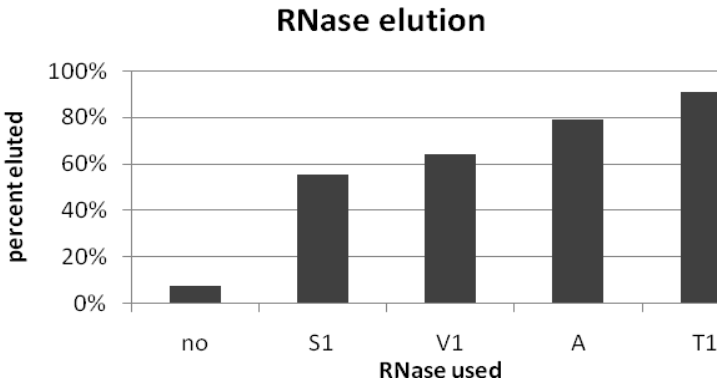

Figure 21: Elution of radioactive EMCV IRES RNA from Streptavidin beads using different RNases (S1, V1, A and T1). Under the conditions tested (A-100, no blocking, incubation at 37 ℃) RNase T1 performed best.

Further optimization of elution was addition of 2 mM Biotin and 5 mM EDTA to the elution buffer and increasing the Mg^{2+} concentration in the wash buffer to 10 mM to freeze complexes and stabilize RNA-protein bounds.
Formation and purification of ITAFs from 48S complexes was verified by western blot against the known ITAF, PABP. PABP could be detected in 48S and RNP complexes (figure 22) formed at the PV and EMCV IRES, but not in complexes formed at the HCV IRES. In contrast to the picornaviral IRES constructs (PV and EMCV), the construct used to assemble complexes at the HCV IRES was not polyadenylated, since HCV does not have a polyA tail, but rather a long and structured 3' UTR (Bung, Bochhaeva et al. 2010).

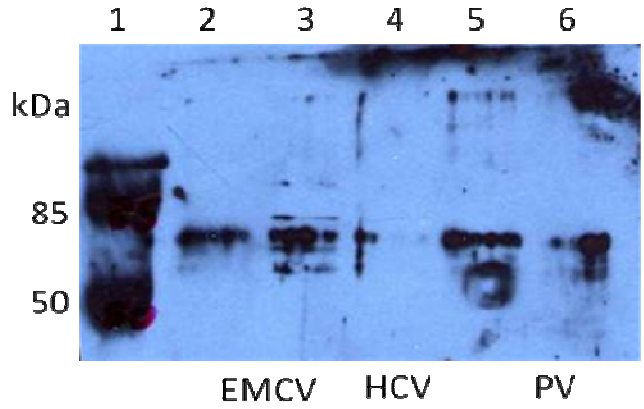

Figure 22: Western blot with an antibody raised against recombinant PABP in rabbit. Translation reactions were assembled in RRL supplemented with 20 % HeLa extract using biotinylated PV-IRES RNA. Then they were either separated into 48S complexes and RNPs on 5 - 20 % sucrose gradients or directly bound to Streptavidin beads as total mRNP complexes.

1: Molecular weight marker

2: 48S peaks formed at the EMCV IRES

3: Total mRNP complexes formed at the EMCV IRES

4: Total mRNP complexes formed at the HCV IRES

5: Total mRNP complexes formed at the PV IRES

6: Total mRNP complexes formed at the PV IRES (no HeLa extract)

Conclusion

Formation of 48S complexes at the PV IRES is rather low in RRL, therefore the protocol was optimized by addition of HeLa extract, m^7GTP and eIF4G(p100). Polyadenylation of the used construct also increased formation of 48S complexes. These 48S complexes were formed using double labeled RNA. Radioactive UTPs were used to identify peaks in sucrose gradients and biotin-UTPs were used to enrich RNA binding proteins from the peaks. A protocol for formation of complexes and enrichment using Streptavidin beads was established.

Proteins bound to the HCV IRES

Translation initiation at the HCV IRES is much simpler than on the PV or EMCV IRESs. 48S complexes can be reconstituted at it just from purified ribosomes and eIF3 without any additional components.

In a translation reaction initiated with only 40S ribosomal subunits and eIF3, 48S initiation complexes form at the HCV IRES but cannot complete translation initiation since 80S subunits, eIF2 and loaded t-RNAs are missing. When incubating those partial translation

reaction using biotinylated HCV RNA and purified components directly with Streptavidin beads, 48S complexes as well as RNPs (eIF3 bound to RNA without 40S subunits) will bind to the beads and can be eluted using RNase. Ribosomal proteins, as well as subunits of eIF3, can be identified on silver stained SDS-PAGE gel (figure 23 and 24). Not biotinylated HCV IRES RNA was used to control for unspecific binding and elution of proteins to the beads. Purified eIF3 (-p170) and pure 40S ribosomal subunits were loaded on the gel for comparison (figure 23, lanes 1 and 9).

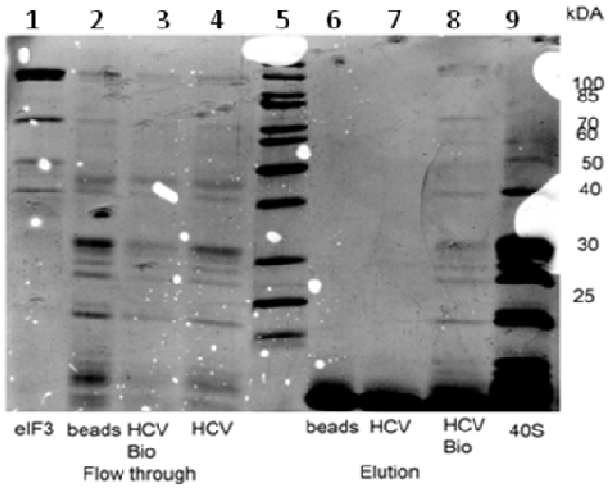

Figure 23: Reconstitution of HCV IRES from purified components (40S ribosomal subunits and eIF3 (-p170)). Initiation reaction was stopped after 15 min by cooling. Then the reaction was incubated with Streptavidin beads and proteins were eluted with RNase A and T1 mix.
beads: beads incubated with extract without RNA (lanes 2 and 6),
HCV: beads incubated with extract containing not biotinylated HCV IRES RNA (lanes 4 and 7),
HCV Bio: beads incubated with extract containing biotinylated HCV IRES RNA (lanes 3 and 8),
pure 40S subunits and eIF3 (-p170) were loaded on the gel as controls (lanes 1 and 8)
Proteins separated on 15 % SDS-PAGE gel and stained using the silver staining protocol described in the methods section.

Since binding and elution from Streptavidin beads was sufficiently efficient I next used extracts to assemble 48S complexes at the HCV IRES. When using extract (RRL + 20 % HeLa) instead of purified component several additional bands can be seen on the gel (figure 24). Especially interesting is the band at ~45kDa present in samples when reconstituted from extract, but not from purified components.

To further investigate the ~45kDa protein found in complexes at the HCV IRES from RRL but not from purified components I performed a sucrose gradient using biotinylated HCV IRES RNA as a template for translation initiation (figure 26 - 28).

Another additional band can be seen at ~72kDa, which seems to be part of the ribosome, since it can be also seen when loading purified 40S ribosomes. It cannot be seen in complexes assembled from pure 40S subunits and eIF3 (-p170). Since silver staining visualizes RNA as well as proteins this band might consist of RNA accumulating at the HCV IRES construct, either from the ribosome or from untreated RRL. This band could not be observed on a coomassie stained gel (figure 25), since coomassie does not stain RNA.

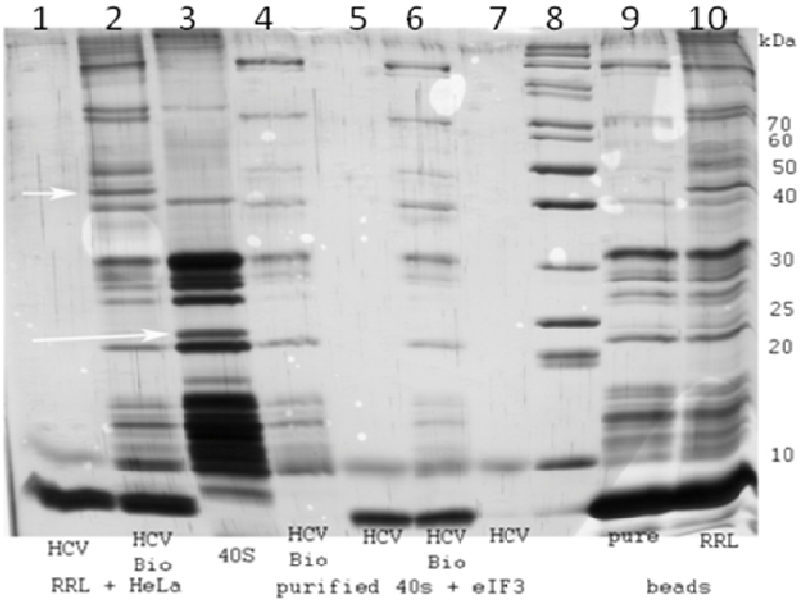

Figure 24: Reconstitution of HCV IRES from purified components (40S ribosomal subunits and eIF3 (-p170), lanes 4-7) and extract (RRL +20 % HeLa, lane 1 and 2). Translation reaction was stopped after 15 min then incubated with Streptavidin beads and proteins were eluted with RNase A and T1 mix. Proteins were separated on a 15 % SDS-PAGE gel and the gel stained using the silver staining protocol.

HCV: not biotinylated HCV IRES, HCV Bio: biotinylated HCV IRES, pure 40S subunits were loaded on the gel as controls (lane 3).

Beads used to purify proteins were eluted a second time by boiling with SDS sample buffer (lanes 9 and 10). Pure: beads from samples seen in lane 4; RRL: beads from samples seen in lane 2.

As shown in figure 24 (lanes 9 and 10) elution from the beads by RNase is incomplete and a second elution using SDS-PAGE sample buffer leads to a much higher yield of proteins as seen in lanes 9 and 10 in figure 24. Boiling with SDS sample buffer however elutes proteins bound unspecifically to the beads as well as disrupting the biotin-Streptavidin bond, therefore eluting RNA and bound proteins. Unfortunately these elutions then include proteins unspecifically bound to the beads and not only RNA binding proteins.

Proteins bound to picornavirus IRESs

As done on the HCV IRES (figure 24), I reconstituted the PV and EMCV IRES using RRL supplemented with 20 % HeLa extract. Addition of m^7GTP and eIF4G (p100) ensured efficient formation of 48S complexes, as described above. Using biotinylated RNA to initiate translation, whole reactions were incubated with Streptavidin beads and proteins eluted using RNase T1 with addition of 2 mM Biotin and 5 mM EDTA. Proteins were separated on 15 % SDS-PAGE Gel and the gel stained with coomassie.

The overall patterns of proteins eluted from translation reaction initiated on all three IRESs are very similar; however there are some slight but important differences (figure 25 lanes 2-5).

The 45 kDa protein observed when translation is initiated at the HCV IRES in RRL supplemented with HeLa (figure 24, lane 2) can be observed here again (figure 25, lane 3). A band with identical size can be seen when initiating at the PV IRES (figure 25, lane 5), but not on the EMCV IRES (figure 25, lane 4). A strong band at ~ 50 kDa may be a picornavirus specific ITAF, since it can only be observed at the EMCV and PV (lane 4 and 5) but not at the HCV IRES (lane 3). Those bands were excised from the gel and sent to the protein analysis facility at Moscow State University for mass-spectrometric analysis.

Another PV specific band was observed at ~100 KDa. It may also be present in EMCV but is much weaker.

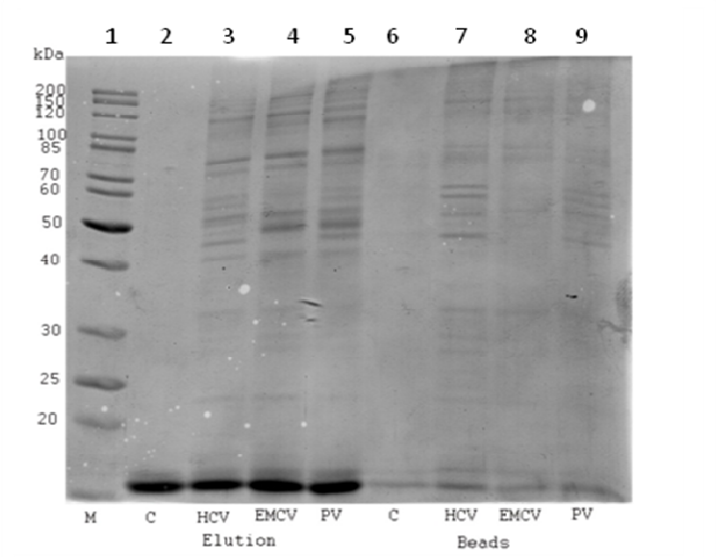

Figure 25: Reconstitution of HCV, EMCV and PV IRESs from extract (RRL + 20 % HeLa),translation initiation reaction in the presence of GMP-PNP was stopped after 15 min by cooling. Then the reaction mixture was incubated with Streptavidin beads and proteins were eluted with RNase T1, as a control not biotinylated PV RNA was used (C). Proteins were separated on a 15 % SDS-PAGE gel and stained with coomassie.

Analysis of proteins using sucrose gradients

To further analyze those protein patterns, radioactive and biotin labeled HCV, PV and EMCV RNA was used to initiate translation. Translation reactions were then separated on 5-20 % sucrose gradients (figure 26).

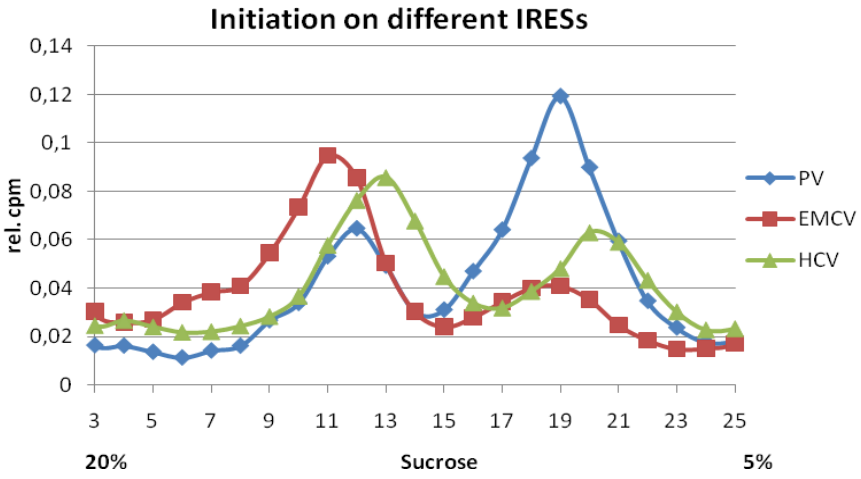

Figure 26: Formation of 48S complexes in an optimized RRL-based system using three different viral IRESs, PV (blue), EMCV (red) and HCV (green). Translation reaction was stopped at the stage of 48S complexes by addition of GMP-PNP and complexes were separated on 5 - 20 % sucrose gradients.

Formation of 48S complexes can be observed with all used IRESs to varying degrees. As expected the EMCV IRES (red line in figure 26) performed best under the used conditions, which were optimized for translation at the related PV IRES. The HCV IRES is approximately 300 nts smaller than the PV and EMCV IRESs resulting in a slight shift of the 48S peak with HCV RNA towards lower sucrose density.

The stability of RNA after the gradient was tested on a 8 % polyacrylamide gel before using the biotinylated RNA to extract proteins from complexes. All three RNAs were reasonably stable with slight degradation after translation initiation and sucrose gradients (figure 27).

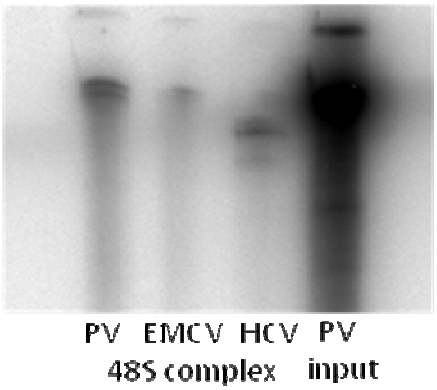

Figure 27: RNA stability in 48S complex tested after 5 - 20 % sucrose gradient and compared to RNA not subjected to extracts and gradient. Radioactively labeled RNAs were separated on a 8 % PA gel and the dried gel used to expose a phosphoimager cassette. The amount of input RNA equals the amount of RNA used in one gradient.

Isolation of proteins from 48S complexes

After verification of RNA quality the biotinylated RNAs from the 48S and RNP peaks (figure 26) were used to extract proteins using Streptavidin beads. Proteins were eluted from beads using RNase T1 and separated on a 15 % SDS-PAGE gel. The band observed in translation reaction at the HCV and PV IRES at 45 kDa (figure 25) can be seen in the 48S complexes (figure 28) although due to different running and staining procedures it appears to be slightly lower (40 - 42 kDa).

Another band at 120 kDa can also be observed in translation reactions at the PV and HCV but not the EMCV IRES. Since the concentration of acrylamide and the running parameters of this gel were optimized for separation of proteins in the range of 30 – 80 kDa, proteins with higher molecular weights were not well separated. I focused on smaller proteins falling into the size range well separated on the gel. Another experiment with different separation conditions could not be performed due to time and material constrains.

The 50 kDa band observed specifically for EMCV and PV (figure 25) is found in the RNP peak, but is absent in the 48S fractions (figure 28), indicating that this protein is not involved in formation of 48S complexes and may even be an inhibitor of translation initiation at picornaviral IRESs. The largest amount of proteins was eluted from the RNP peak formed at the PV IRES, due to it being the largest peak of the gradient (figure 26). This may be the reason why two PV specific bands at 100 and 125 kDa can be observed in the RNP peak but not in the 48S peak. Unfortunately the bands detected in the 48S peaks assembled on all three IRESs proofed too weak for mass-spectrometric analysis.

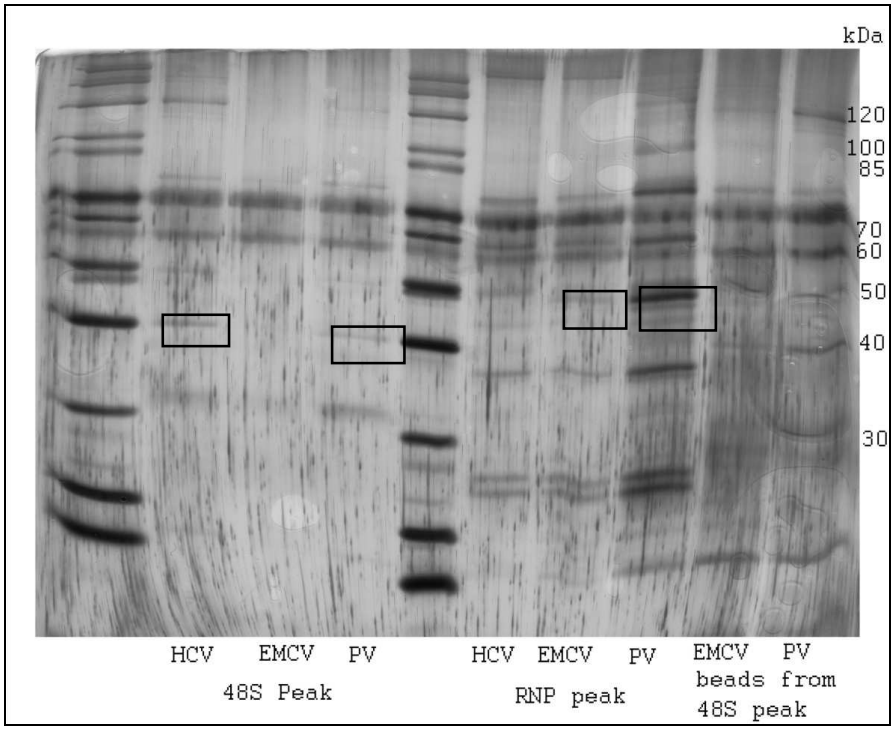

Figure 28: Separation of proteins extracted from sucrose gradient (figure 26) on a 15 % SDS-PAGE gel and stained using the silver staining protocol. Peak fractions from the 48S and RNP peak respectively were combined and incubated with Streptavidin beads, proteins were eluted from beads using RNase T1 and 2 mM biotin. Proteins (p45 and p50) chosen for further analysis are indicated by black squares.

However since the same bands were observed on the gel prepared from total complexes at all three IRES (figure 25), the bands from the first gel were excised and sent for mass-spectrometric analysis.

Results of mass spectrometry

Band excised from coomassie stained SDS-PAGE gels (figure 25) were sent to the Mass spectrometry facility at Moscow State University. The results were analyzed using MASCOT at http://www.matrixscience.com. In total 4 bands were sent for mass spectrometry, p45 from the 48S peak of translation reactions initiated at the PV and HCV IRES and p50 from the RNP peak of translation reactions initiated at the PV and EMCV IRES (figures 25 and 28).

P45 was in both cases identified as cytoplasmic actin, a precursor of actin fibers in the cytoskeleton. Since actin is one of the most abundant proteins in the cell a contamination of the samples is rather likely. Separation of the band observed at 45 kDa on a 2D-Gel could allow further insight into its composition. It is possible, even likely that another protein of the same size can be detected there.

P50 was in both cases identified as $ITAF_{45}$/PA2G4/EbP1 a known ITAF for FMDV, which has not been described for PV or EMCV. $ITAF_{45}$/PA2G4/EbP1 stimulates translation at the FMDV IRES (Monie et al. 2007) and was shown to interact with the EMCV IRES, but not to stimulate it (Bonnal et al. 2005). The fact that $ITAF_{45}$/PA2G4/EbP1 was detected in the RNP peak, but not in the 48S peak, indicates binding of $ITAF_{45}$/PA2G4/EbP1 to the IRESs but not necessarily stimulation of formation of 48S complexes.

$ITAF_{45}$/PA2G4/EbP1 is in uninfected cells a transcription factor probably involved in rRNA processing (Strausberg et al. 2001, Squatrito et al. 2004) but is known to shuttle between nucleus and cytoplasm (Squatrito et al. 2004), where it interacts with the ErbB3 receptor (Zhang et al. 2008). EbP1 was implicated in growth regulation (Squatrito et al. 2004) and translation initiation via phosphorylation of eIF2.

However, the influence of both factors or at least of p50 in PV translation needs to be tested by functional assays using recombinant or purified protein in an in vitro translation system like RRL.

Conclusion

48S complexes were formed at the PV, HCV and EMCV IRES using double labeled RNAs. Using the biotin-tag proteins bound to the RNAs could be identified. A picornavirus-specific protein, EbP1, was identified in the RNP peak at the PV and EMCV IRES. Another factor, cytoplasmic β-actin, was identified at the PV and HCV IRES exclusively in 48S peaks.

Depletion of HeLa extract

Poliovirus does not translate well in the in vitro translation system RRL. Supplementing RRL with HeLa extract leads to a significant increase in translation of reporter mRNAs under the control of the PV IRES (Dorner et al. 1984). This effect is due to ITAFs present in the extract.

Two of them have been identified (La and unr), but there are most likely others. Using internally biotinylated PV IRES RNA coupled to magnetic Streptavidin beads I successfully depleted HeLa extract of RNA binding proteins (figure 29) to identify proteins necessary for the stimulatory effects of HeLa extract.

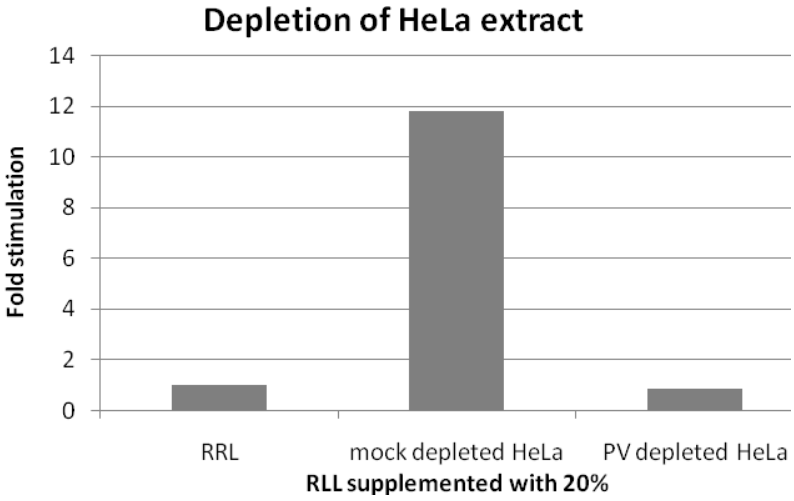

Figure 29: Translation reaction of a PV IRES Luciferase reporter in RRL supplemented with 20 % Hela extract depleted of RNA binding proteins. Depletion was achieved either using the PV IRES bound to Streptavidin coupled magnetic beads or just beads (mock depleted). Luciferase activity was determined and compared to activity in not supplemented RRL.

After successful depletion I tried to restore the stimulatory activity of HeLa extract by adding proteins, which were eluted from the beads used to deplete the extract. Those were eluted by digestion with micrococcal nuclease, since this nuclease can be easily inhibited by addition of EGTA. Unfortunately addition of elution fractions did not restore the stimulating activity of HeLa extract (figure 30). I hypothesized that proteins bound only weakly to the RNA via protein-protein interactions might be washed off during the procedure, so I added the washes to RRL, which also did not result in stimulation (figure 30). Next I combined washes and elution fractions, since RNA-binding ITAFs like La are expected to be present in the elution but not in the washes. Even a combination of washes with elution did not stimulate translation at the PV IRES in RRL (figure 30).

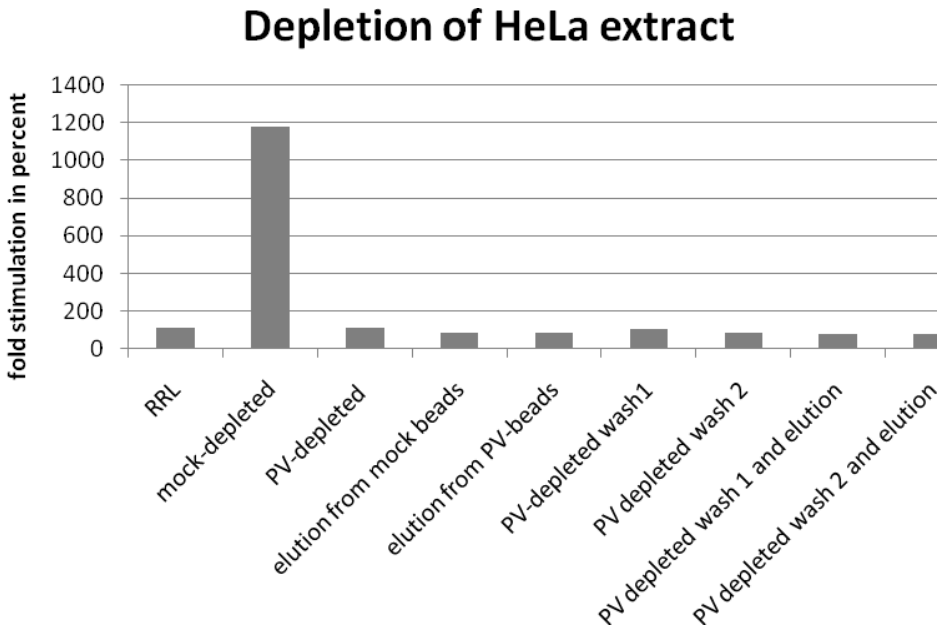

Figure 30: Translation reaction with the PV IRES luciferase construct in RRL supplemented with 20 % Hela extract depleted of RNA binding proteins. Depletion was achieved with the PV-IRES bound to Streptavidin coupled magnetic beads or just beads (mock depleted). Additionally RRL was supplemented with 20 % elutions and washes from beads used to deplete. Luciferase activity was determined and compared to activity in not supplemented RRL in percent.

The stimulating activity of HeLa extract on translation under the control of the PV IRES could be depleted by binding this activity to PV IRES coupled beads. The activity could not be restored by elutions from the beads. It is possible that this factor is not an RNA binding protein but a factor that facilitates binding of other ITAFs to the IRES, as described for SrP20 (Bedard et al. 2007) and NF45 (Merrill & Gromeier 2006). Most likely however the stimulating factor together with the known ITAFs unr and La could not be eluted from the beads using RNase. In conclusion this approach did not yield any new hints on the nature of the missing ITAF(s).

Influence of polyA tail on initiation of translation

During optimization of translation initiation to identify proteins bound to the PV IRES, I discovered that addition of 50As to the 3' UTR of an mRNA controlled by the PV IRES has a pronounced effect on formation of 48S complexes on the PV IRES but not the EMCV IRES (figure 18). Stimulation of translation by polyA has been known for a long time (Gallie 1991,

Bergamini et al. 2000 and figure 16), but the exact mechanisms are still to be elucidated. The effect may be due to protection of the mRNA from degradation, ribosome recycling or a signal about mRNA 3' end stability to the 5' end by induction of conformational changes in initiation factors. Using GMP-PNP to stop ribosomes at the 48S stage I discovered a pronounced effect of the polyA tail on this early step of translation (Figure 17 and 18).
To control if this effect may be an artifact resulting from the use of GMP-PNP I used a different inhibitor, anisomycine, which stops translation at the 80S stage (figure 31).

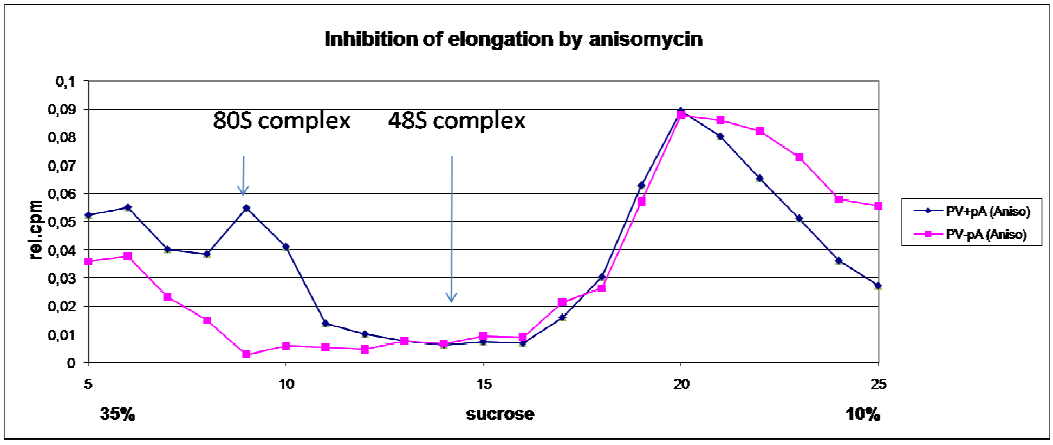

Figure 31: Translation initiation at the PV IRES with (diamonds) and without (squares) addition of 50 As to the 3' end of the RNA, translation reaction were stopped at the stage of 80S ribosomes by addition of anisomycine and separated on 10-35 % sucrose gradients.

With anisomycin as inhibitor the stimulation by $polyA_{50}$ tail could be seen even more pronounced as when using GMP-PNP (compare figures 17 and 31). I was not able to detect any peaks in sucrose gradients when not accumulating complexes by addition of inhibitors since PV initiates translation rather inefficiently in RRL, even when supplemented with 20 % HeLa extract. Artificial effects of the inhibitors can therefore not be excluded.
To further investigate the mode of action by polyA tails I compared the amount of 48S complexes formed at different time points, from 30 s to 10 min, in the presence of GMP-PNP to stall 48S complexes. Translation initiation was stopped at the appropriate time point by addition of unphysiological amounts (30 mM) of $MgCl_2$ which stabilizes existing complexes and prevents empty ribosomal subunits from binding to RNA (figure 32).

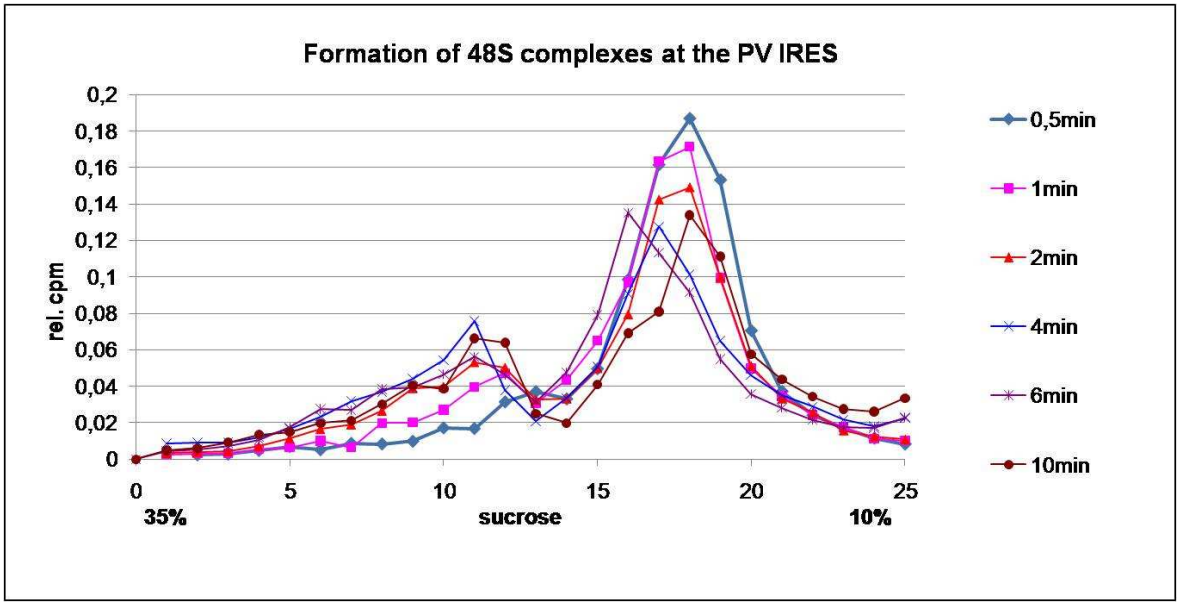

Figure 32: Formation of 48S complexes at the PV IRES over ten minutes; subunit joining was inhibited by GMP-PNP. Formation of 48S complexes was stopped at appropriate time points by addition of 30 mM $MgCl_2$ and immediate cooling of the reaction to 0 ℃. Tra nslation reactions were separated on 10-35 % sucrose gradients.

Three independent repeats of the time course experiment with and without $polyA_{50}$ were performed and results combined in figure 33. The results indicate that formation of 48S complexes in both cases, with and without $polyA_{50}$ is complete after 2 minutes and that on RNA with polyA tail more 48S complexes can form (figure 33). Since I used GMP-PNP to stall 48S complexes, only one complex can form per RNA molecule. Equal amounts of RNA were used, so the higher amount of radioactivity accumulating in the 48S peak with polyA indicates that formation of 48S complexes is much more efficient with polyA. Formation of 48S complexes seems to be more efficient when using polyA tails, as seen in figure 17, where the amount of mRNPs decreases with addition of polyA tails. In this system, supplemented treated RRL, ribosomes and initiation factors are limited, and are more efficiently connected to RNAs with polyA.

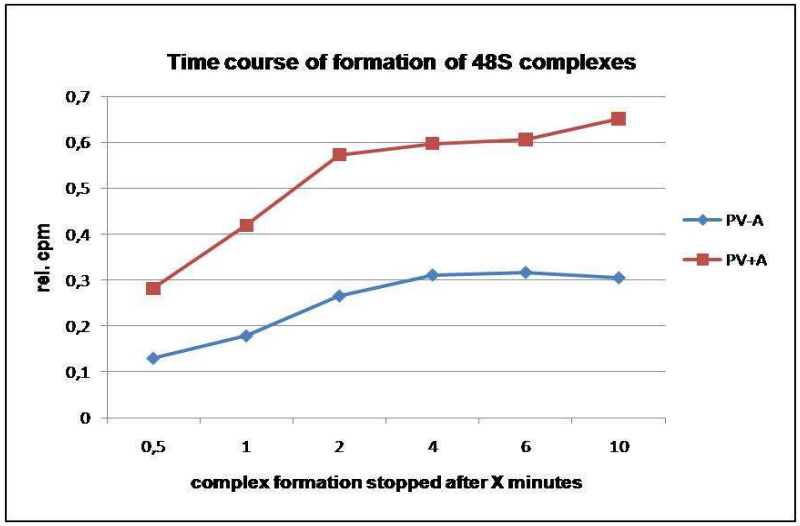

Figure 33: Summary of three independent time course experiments initiating translation at the PV IRES with (squares) and without (diamonds) addition of 50 As. Subunit joining was inhibited using GMP-PNP and separated on 5 - 20% sucrose gradients. Rel.cpm in 48S complexes was calculated using the area under the curve in 48S complexes.

Conclusion

PolyA tails have a significant effect on translation initiation at the PV IRES, but not at the closely related EMCV IRES. With polyA tails significantly more initiation complexes form, while the rate of formation is not influenced.

UV-crosslinking of La to the PV IRES

As outlined in the proposal of my project the purpose of purifying eukaryotic initiation factors (eIFs) and ITAFs was to further investigate their binding to the PV IRES and interaction with each other while binding using UV-crosslinking. Purification of eIFs took longer than anticipated in the plan of the PhD project and other approaches to find the missing ITAFs were tried first as described above. Consequently only very few crosslinks were performed; investigating the synergistic effects between the La autoantigen, PTB and eIF4A.

A short radioactively labeled RNA was synthesized by in vitro transcription consisting of the PV IRES and 250 nt of the luciferase ORF. The construct was incubated with pure protein preparations in different combinations. Proteins bound to the RNA were irreversibly crosslinked to U-residues by UV light.

The antiautogen La is cross-linked to the PV IRES when incubated with PV RNA by itself (figure 34), but not when incubated in direct competition with PTB or recombinant eIF4A. In

mix with PTB La binding to the IRES is barely detectable, while it is completely absent in an equimolar La/eIF4A mixture.

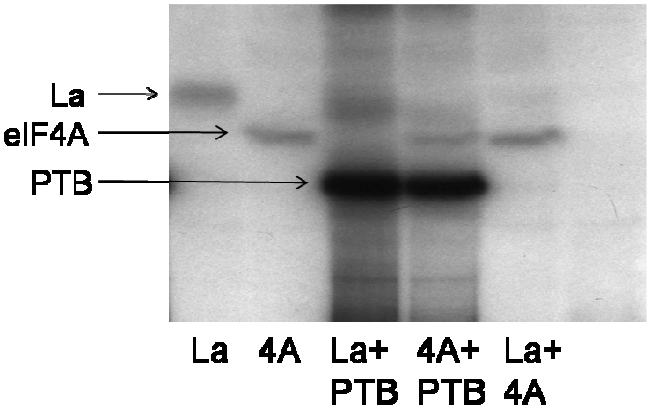

Figure 34: UV-crosslinking of purified recombinant La, eIF4A and PTB to PV IRES RNA. Different equimolar combinations of proteins were tested. Crosslinking was performed for 30 min with UV light of the wavelength 320 nm and proteins separated on 12 % SDS-PAGE. X-ray film was exposed to the dried gel for 3 days at 4℃.

La binding to the PV IRES is strongly influenced by other factors binding at the same position (PTB) or changing the secondary structure of the IRES (eIF4A). To further investigate La binding to the IRES different concentrations of La and competitors need to be tested. Interactions between ITAFs are very interesting but hard to investigate in purified systems, since interactions in vivo are most likely very different from interactions in vitro or with purified components.

Meanwhile, however, the search for new ITAFs continued and maybe the elusive factor is not a protein after all.

Effects of miRNAs on translation initiation at the PV IRES

Identification of neuron-specific miRNAs

miRNAs, which potentially bind to the PV IRES were identified using ViTa (www.vita.mbc.nctu.edu.tw accessed on 18.6.2008). ViTa uses the algorithms of Miranda and targetscan (Lewis et al. 2003) on a database which is compiled from miRNBase, miRNA-Map, ICTVdB, VBRC and VirGen. The output of ViTa is an interactive graphic (figure 35), which due to its interactivity cannot be displayed in all details here. For clarity the results are compiled in the following table showing all miRNAs identified to bind to the IRES-region of the PV genome.

miRNA	Binding site	Specificity	Sequence	Literature
18b	4-27	placenta	5'- UAAGGUGCAUCUAGUGCAGUUA -3'	O'Donnell et al. 2005
20a	149-172	bladder	5'- UAAAGUGCUUAUAGUGCAGGUAG -3'	Lagos-Quintana et al. 2001
107	**446-465**	**brain, kidney**	**5'-** AGCAGCAUUGUACAGGGCUAUCA **-3'**	**Mourelatos et al. 2002**
127	**209-227, 517-540**	**brain, placenta**	5'-UCGGAUCCGUCUGAGCUUGGCU-3'	**Lagos-Quintana et al. 2002**
130b	**266-288**	**placenta, brain**	**5'-** CAGUGCAAUGAUGAAAGGGCAU **-3'**	**Weber et al. 2005**
210	503-523	lung	5'- CUGUGCGUGUGACAGCGGCUGA -3'	Lim et al. 2003
221	294-312	prostate	5' AGCUACAUUGUCUGCUGGGUUUC 3'	Lim et al. 2003
301	**270-288**	**brain**	**5'** CAGUGCAAUAGUAUUGUCAAAGC **3'**	**Lu et al. 2005**
302a	152-171	stem cells	5' UAAACGUGGAUGUACUUGCUUU 3'	Weber et al. 2005
324-5p	**3-22**	**brain, prostate, placenta**	5' CCACUGCCCCAGGUGCUGCUGG 3'	**Kim et al. 2004**
326	**2-12 297-304**	**brain, neuronal**	5' CCUCUGGGCCCUUCCUCCAG 3'	**Kim et al. 2004**
346	**348-373**	**brain**	5' UGUCUGCCCGCAUGCCUGCCUCU 3'	**Kim et al. 2004**
422a	**505-529 (874-903)**	**brain, muscle**	5' CUGGACUUAGGGUCAGAAGGCC 3'	**Kasashima et al. 2004**
422b	505-529 (874-903)	muscle	5' CUGGACUUGGAGUCAGAAGGCC 3'	Kasashima et al. 2004
452*	308-329	placenta	5' UCAGUCUCAUCUGCAAAGAAG 3'	Bentwich et al. 2005

Table 2 : miRNAs identified to bind the PV IRES (nt 1-793), obtained from ViTA www.Vita.org on 18.6.2008. Information about specificity were obtained using the miRNbase at www.microrna.org *on 19.6.2008 and on 26.1.2010.* Brain-specific miRNAs are indicated in bold, miRNAs chosen for further studies are indicated in red.

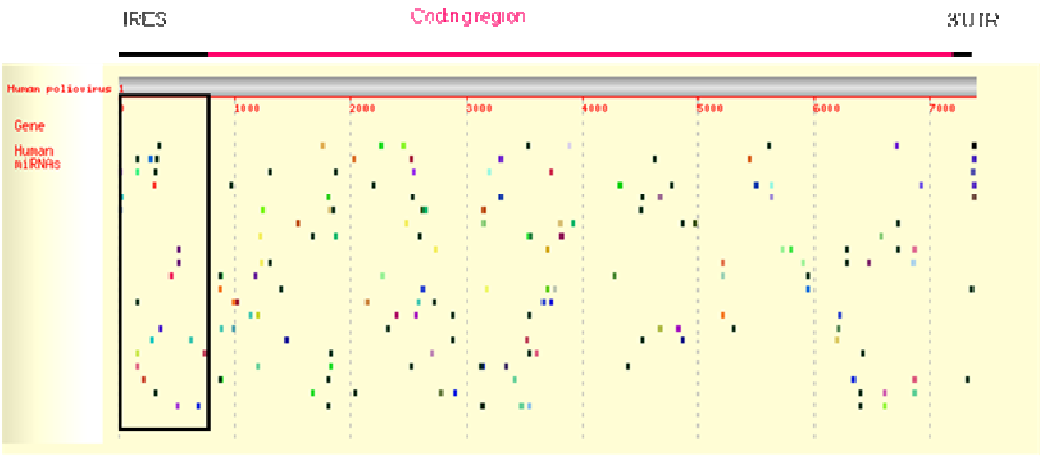

Figure 35: Graphic output of ViTa depicting all miRNAs bound to the poliovirus genome as colored dots with no apparent colorcode. On the ViTa website each dot represents a link to another page, which contains further information about this particular miRNA and its binding site.
The IRES region is marked with a black square. All miRNAs present in the black square are listed in table 2.

Three miRNAs, miRNA-127, 326 and 422a were chosen for their binding sites and exclusive neuron specificity (table 2 and figure 35). Their binding sites were located in the structural model of the PV-IRES based on work by Pilipenko et al. (1989) (figure 36). Both miRNA-326 and miRNA-422a bind to the IRES in predicted loop structures, while the predicted miRNA-127 binding site is located in a paired region in the stem of loop III.

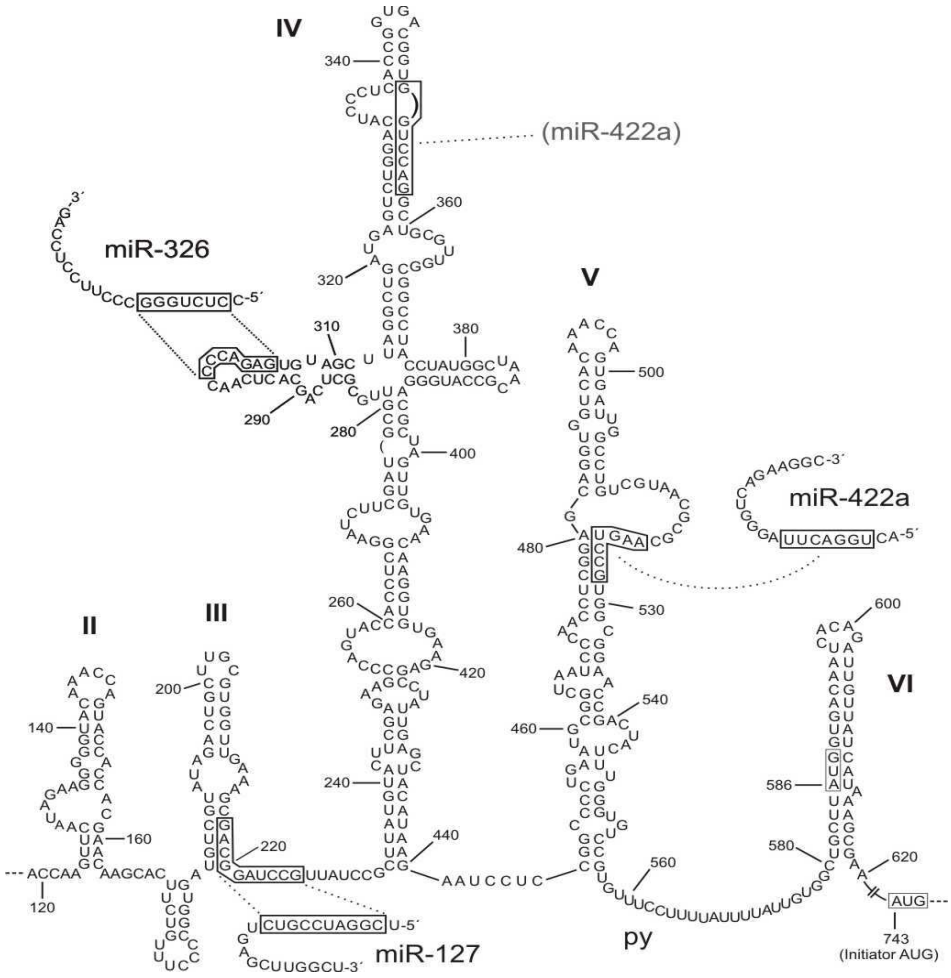

Figure 36: Computer-based model of PV IRES modified from Pilipenko et al. (1989); miRNA-binding sites are indicated. Stem loop I with another binding site for miRNA-326 is missing from this model since it is not part of the IRES.

The chosen miRNAs were further characterized using Miranda and other miRNA databases. miRNA-127 was identified in humans using northern blot and RT-PCR by Lagos-Quintana et al. (2002), where it was described as neuronal. However, Landgraf et al. (2007) showed a more ubiquitous distribution for miRNA-127 using more sensitive sequencing and blotting techniques. It is especially abundant in spleen (Kelly & Russel 2009). miRNA-127 was mapped to an intergenic region on chromosome 14. Both strands of the duplex are recognized miRNAs, miRNA-127-3p and miRNA-127-5p. The miRNA-127, which has a binding site at the PV IRES is miRNA-127-3p. miRNA-127 is a mammalian-specific miRNA, no homologues can be found in *Drosophila melanogaster* or *Xenopus laevis* (Friedman et al. 2008). The natural targets of miRNA-127 are among others a mRNA coding for a lysine methyltransferase and the mitogen-activated kinase 4 (MAPK4).

miRNA-326 was identified as neuron specific in rats and mice (Kim et al. 2004) and identified in humans using computer-based genome searches, which were later verified experimentally (Landgraf et al. 2007). It was mapped to chromosome 11, where it is expressed in intron 1 of beta-arrestin, a protein involved in desensitation of G-coupled receptors. In rat brain miRNA-326 is especially abundant during neuronal growth, indicating a developmentally regulated expression and activity pattern. Its natural targets are diverse mRNAs, whose proteins are mostly involved in growth and expression regulation, like the orthopedia homeobox gene and MAPK4. miRNA-326 also affects mRNAs of proteins involved in PV translation, such as the known ITAF PTB and eIF4B.

miRNA-422a was first identified via computer-based genome analysis as a paralogue of miRNA-422b in humans and later verified experimentally and mapped to an intergenic region on chromosome 15 (Landgraf et al. 2007). Its natural targets are mRNAs coding for e.g. polyA polymerase A and the H3 Histone.

Effect of miRNA-326* on luciferase reporter in RRL

All identified neuronal miRNAs were ordered from biomers.net. For each duplex the guide (mat) and the passenger strand (*) were synthesized by biomers.net. Both strands were then tested independently as single stranded RNAs in the in vitro system RRL and as duplex in HeLa and SH-SY5Y cells.

All miRNAs were tested with the pMPolio construct (Ochs et al. 2003), consisting of the PV IRES and the firefly luciferase (deWet et al. 1997), and pD5 which contains the firefly luciferase with a short vector-derived 5' UTR between the T7 promoter and the beginning of the ORF (figure 37).

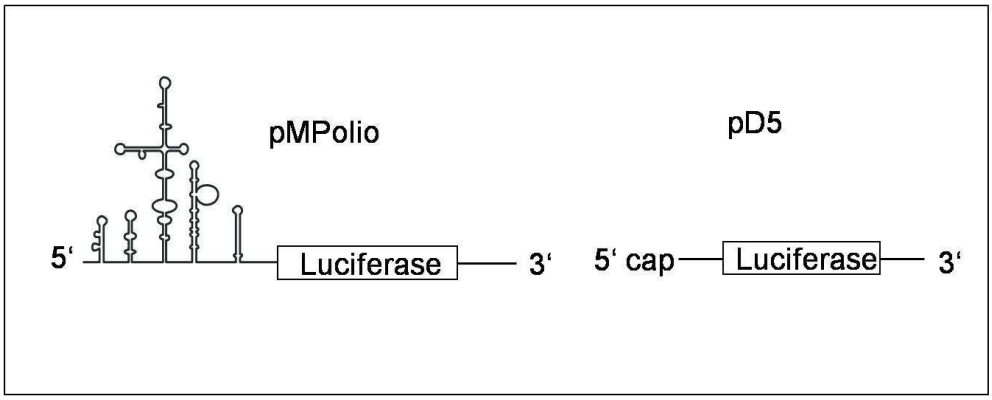

Figure 37: RNA constructs used for in vitro translation and transfections. pD5 was capped for transfection but not for in vitro translation. DNA templates were derived from plasmids by digestion and RNA was transcribed in vitro using SP6 polymerase.

Results obtained in vitro using RRL were inconclusive. Only addition of miRNA-326* showed a significant dose-dependent decrease in translation efficiency (figure 38). However this inhibiting effect could also be seen using the pD5 control (figure 39) and is therefore most likely not an effect of the miRNA-326* on the PV IRES, but an effect of this miRNA on the luciferase reporter.

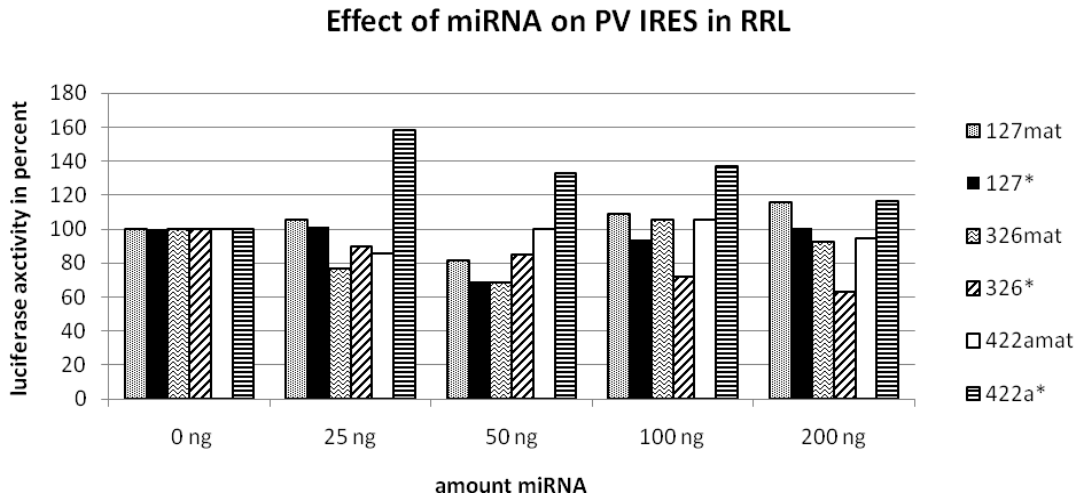

Figure 38: Influence of ss miRNAs on translation of the PV IRES luciferase construct in RRL. All values are depicted in relation to activity with no addition of miRNA which is set to 100 %. An inhibiting effect of miRNA-326* can be seen (waves). The experiment was performed twice in duplicates therefore no standard deviation can be calculated.

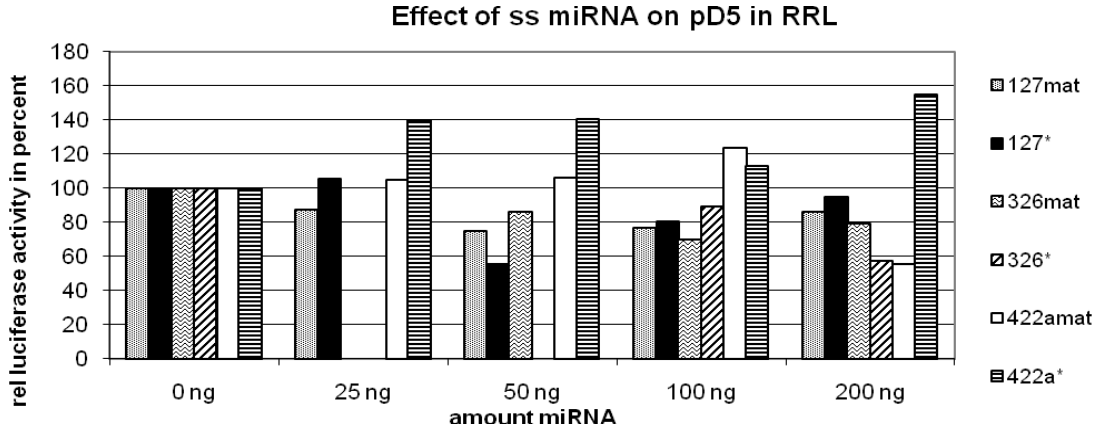

Figure 39: Effect of ss miRNAs on translation of the pD5 luciferase control construct in RRL. All values are depicted in relation to translation without addition of miRNA which is set to 100 %. An inhibiting effect by miRNA-326* can be seen here as well (waves) as in figure 36. Values are missing for some miRNAs when experiments were not performed at these concentrations. The experiment was performed twice in duplicates therefore no standard deviation can be calculated.

This suspected effect of miRNA-326* on the reporter was further investigated and confirmed by the inhibition of the pD5 reporter by miRNA-326 duplex when transfected into HeLa cells (figure 40).

There will be a more detailed discussion of the effect of miRNA-326* on the reporter in a later chapter.

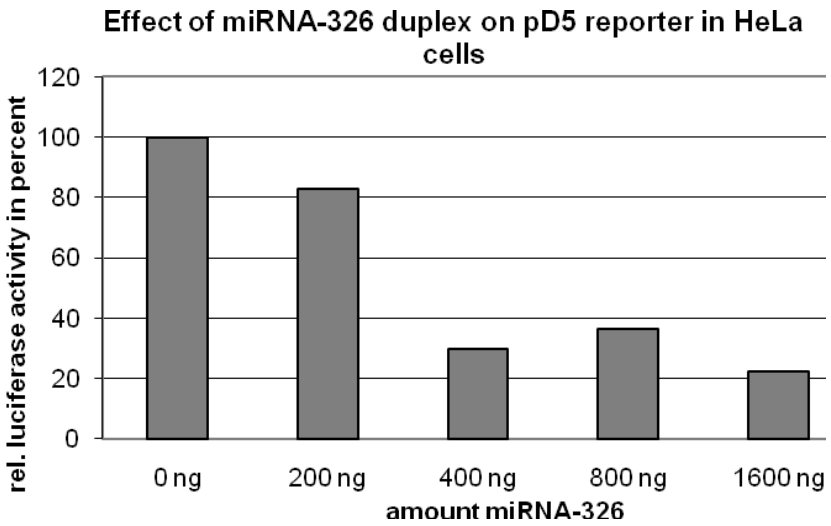

Figure 40: Influence of miRNA-326 duplex on translation of the pD5 luciferase control construct when co-transfected into HeLa cells. All values are depicted in relation to reaction without addition of miRNA which is set to 100 %. The experiment was performed twice in duplicates therefore no standard deviation can be calculated.

Effects of neuronal miRNAs on translation initiation at the PV IRES in cell lines

Since it was shown that RRL is not a good system to study PV (Svitkin et al. 1996) and miRNA-interaction in general (Fabian et al. 2009, Goergen & Niepmann unpublished results), characterization of miRNA effects on the PV IRES was undertaken in cell lines. Two cell lines were chosen for this study, HeLa cells and the neuroblastoma cell line SH-SY5Y (LaMonica & Racaniello 1989).

I used RNA transfection to avoid artifacts stemming from transcription (e.g. via cryptic promoters) and cellular responses to artificial DNA in the cytoplasm and nucleus. Poliovirus is a (+) strand RNA virus (Semler et al. 1984); its (+) ss RNA genome serves as a template for translation in the cytoplasm of the infected cell. Thus it is a functional mRNA upon infection

without the need for nuclear events for successful translation initiation. Also miRNAs normally act on mature mRNA in the cytoplasm. Thus transfection of RNA is both more natural and convenient, in particular since miRNAs can be transfected together with its desired target mRNA (Lytle et al. 2007).

miRNA-duplexes were generated from in vitro synthesized single stranded miRNAs (biomers.net) to be used in transfection experiments. The double stranded RNAs mimic cleavage products of DICER and can be incorporated into the RISC (Lytle et al. 2007).

miRNA-127

miRNA-127 duplex and PV IRES luciferase RNA were transfected into SH-SY5Y cells using Lipofectamine 2000™ and luciferase activity was determined 4 h after transfection. Luciferase values were normalized to metabolic activity as a measure of cell viability determined by the WST1 test.

No significant effect of miRNA-127 on translation at the PV IRES could be detected (figure 41).

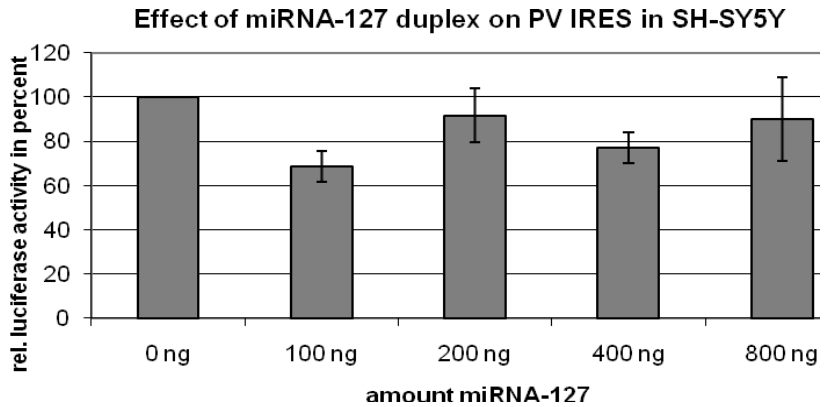

Figure 41: Effect of miRNA-127 on translation of the PV IRES luciferase construct when transfected in SH-SY5Y cells. All values are depicted in relation to reactions without addition of miRNA which is set to 100 % and normalized to cell viability measured by WST1. The experiment was performed three times in duplicates.

miRNA-422a

The influence of miRNA-422a on translation on the PV IRES was examined in cultured cells only very briefly since the results in RRL and computer-based analysis of the firefly luciferase coding region (figure 49) hinted a strong effect of miRNA-422a on the firefly luciferase reporter.

The inhibiting effect can be seen in cells (figure 42) as well as rather weakly in RRL (figure 38 and 39). miRNA-422a inhibits translation of the PV IRES (figure 38 and 42, 43) as well as of the pD5 reporter (figure 39 and 44). Therefore miRNA-422a was excluded from further studies.

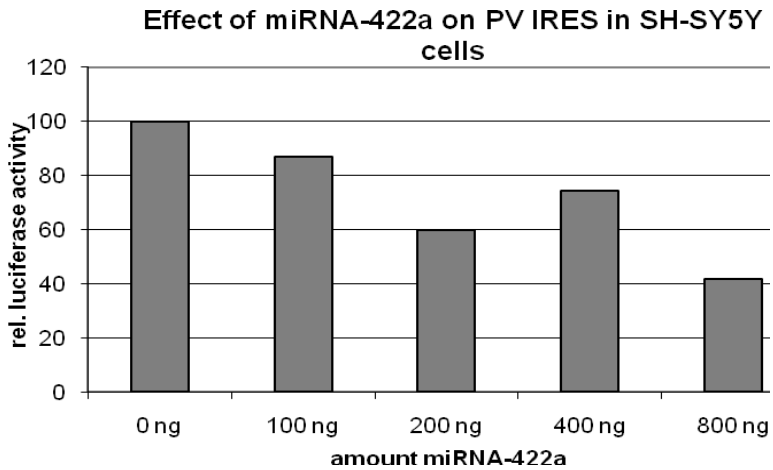

Figure 42: Effect of miRNA-422a on translation of the PV IRES luciferase mRNA when transfected in SH-SY5Y cells. All values are depicted in relation to translation without addition of miRNA which is set to 100 %. Depicted values are the result of two independent experiments performed in duplicates, therefore no standard deviation can be calculated.

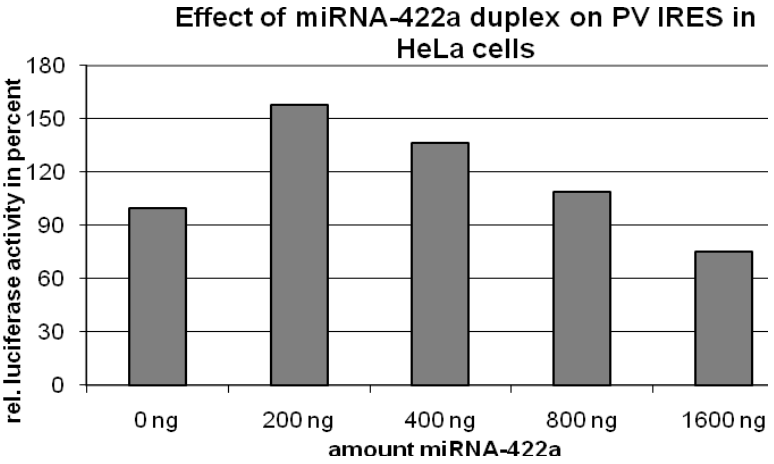

Figure 43: Effect of miRNA-422a on translation of the PV IRES luciferase mRNA when transfected in HeLa cells. All values are depicted in relation to translation with no addition of miRNA which is set to 100 %. Depicted values are the result of two independent experiments performed in duplicates, therefore no standard deviation can be calculated.

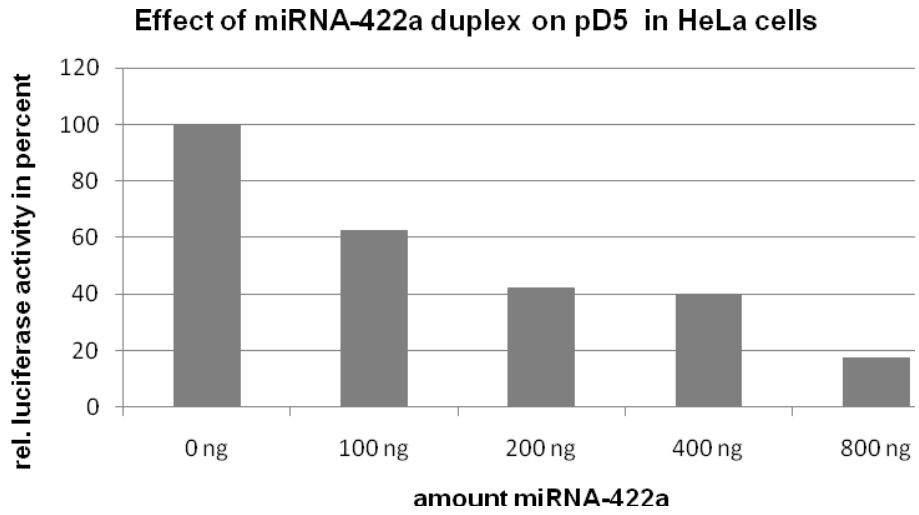

Figure 44: Influence of miRNA-422a on translation of the pD5 luciferase control mRNA when transfected in HeLa cells. All values are depicted in relation to reaction without addition of miRNA which is set to 100 %. The experiment was performed once in duplicates.

miRNA-326

miRNA-326 duplex was transfected into HeLa and SH-SY5Y cells and luciferase activity was determined. miRNA-326 stimulates translation on the PV IRES in both cell lines slightly but significantly (figure 45 and 46), even though the inhibitory effect on the pD5 reporter can be detected in cells (figure 40) and can be assumed to be present in the PV IRES-firefly luciferase construct as well.

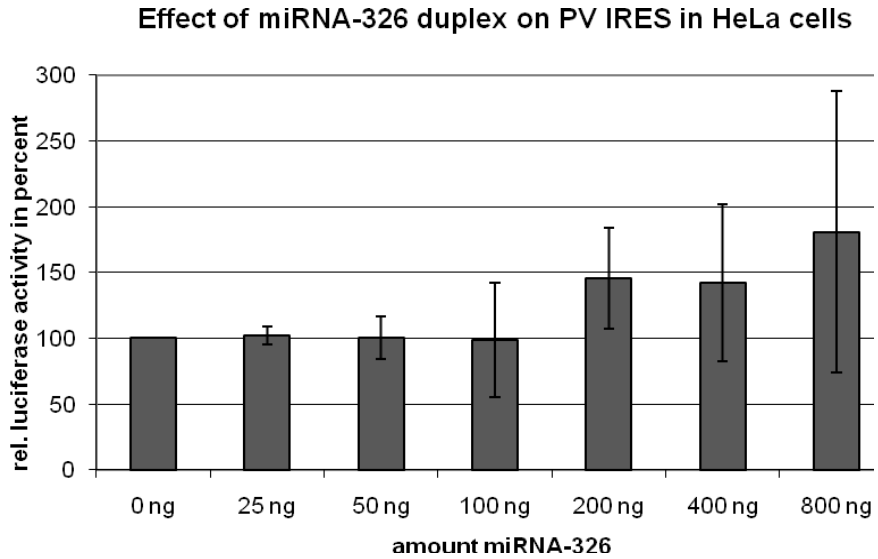

Figure 45: Effect of miRNA-326 on translation of the PV IRES luciferase construct when transfected in HeLa cells. All values are depicted in relation to translation reaction without addition of miRNA which is set to 100 %. Depicted values are the result of five independent experiments performed in duplicates.

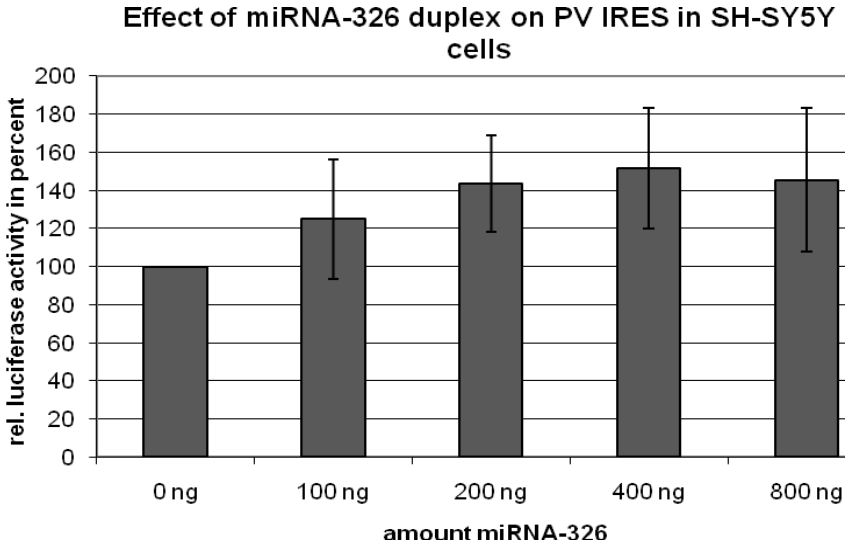

Figure 46: Effect of miRNA-326 on translation of the PV IRES luciferase mRNA when transfected in SH-SY5Y cells. All values are depicted in relation to reactions without addition of miRNA which is set to 100 %. Depicted values are the result of three independent experiments performed in duplicates.

Mutation of the miRNA-326 binding site in stem loop IV of the PV IRES

To confirm that the stimulating effect of miRNA-326 duplex is due to a genuine miRNA effect rather than an unspecific effect resulting from the presence of exogenous double stranded RNA in the cell, the binding site for miRNA-326 in the PV IRES was mutated using site-directed mutagenesis. Care was taken not to interrupt the loop structure of the IRES at this point by avoiding introducing pairing nucleotides.

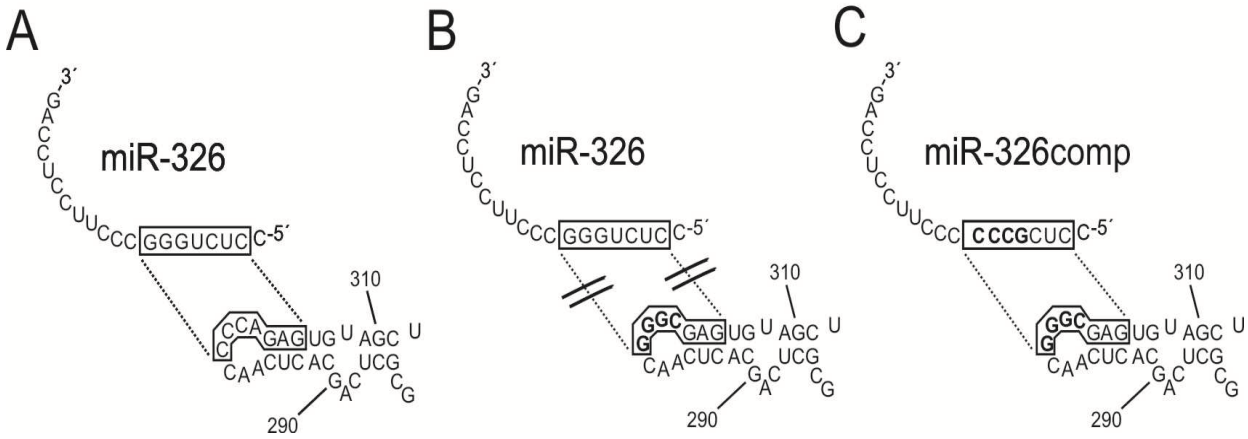

Figure 47: Mutation introduced into stem loop IV of the PV IRES by site-directed mutagenesis (B). The miRNA-326 binding site (CCCAGAG) was replaced by a not pairing sequence (GGGCGAG). Care was taken not to interrupt the loop structure by introducing pairing nucleotides. A complementary mutated seed sequence was introduced into miRNA-326 (C).

The stimulating activity of miRNA-326 could not be observed with the mutated construct (figure 47B and 48) indicating a specific effect of miRNA-326 rather than an unspecific effect by ds RNA.

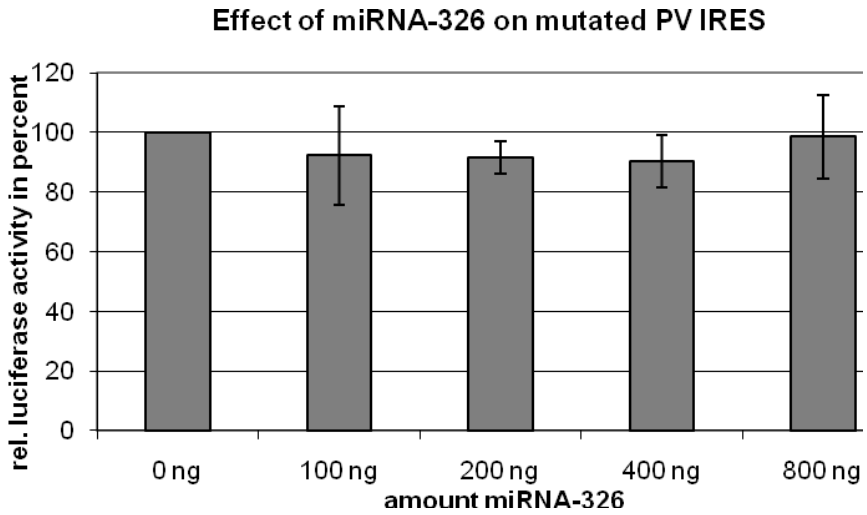

Figure 48: Effect of miRNA-326 on translation of the PV IRES luciferase mRNA with the binding site for miRNA-326 in the IRES mutated. RNAs were transfected into HeLa cells. All values are depicted in relation to reactions without addition of miRNA which is set to 100 %. Depicted values are the result of three independent experiments performed in duplicates.

A compensatory mutation was included in the miRNA (miRNA-326 comp, figure 47C) and the mutated miRNA transfected into HeLa cells together with the mutated IRES.
The observed effect was a dose-dependent stimulation of about 1.4 fold at highest miRNA-326 comp concentrations. This stimulation is not as pronounced as the effect observed with the wild type IRES stimulated by wild type miRNA-326 in HeLa cells. However, the miRNA-326 binding site mutant in the PV IRES could at least be partially rescued by addition of complementary mutated miRNA-326 (miRNA-326 comp).

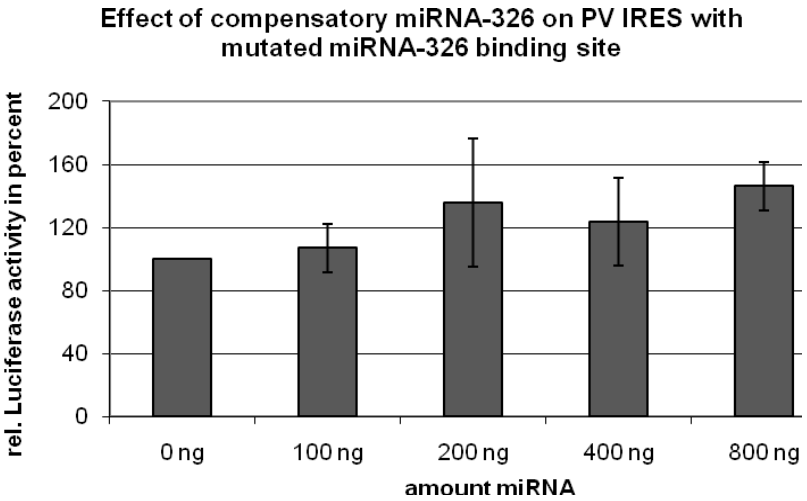

Figure 49: Effect of miRNA-326comp on translation of the PV IRES luciferase mRNA with the binding site for miRNA-326 mutated. RNAs were transfected into HeLa cells. All values are depicted in relation to reactions without addition of miRNA which is set to 100 %. Depicted values are the result of four independent experiments performed in duplicates.

However, the mutated PV IRES showed a much lower overall translational activity than the wild type PV IRES in initiating translation of the reporter protein (figure 50).

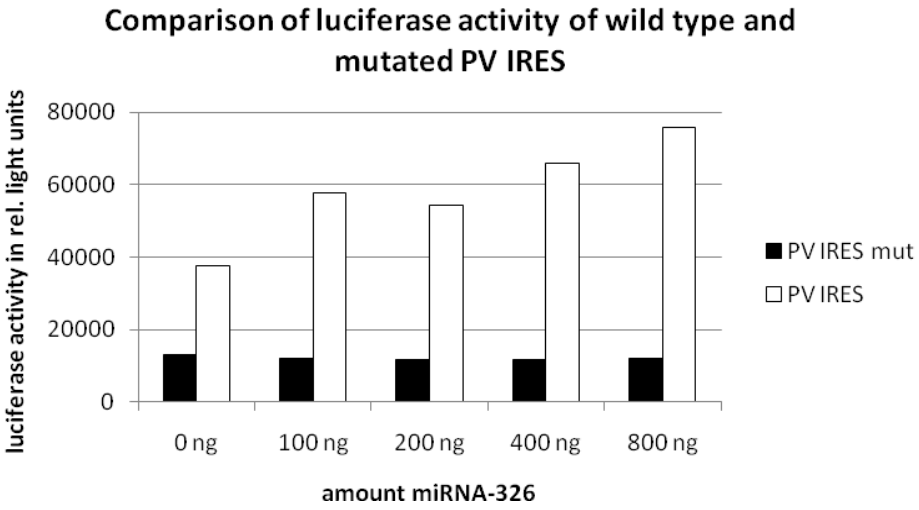

Figure 50: Effect of mutation in the PV IRES (miRNA-326 binding site) on translation efficiency of the PV IRES luciferase mRNA co-transfected with miRNA-326. Equal amounts of RNAs were transfected into HeLa cells.

Since the miRNA-326 binding site in stem loop IV (figure 47) is identical with a binding site for PCBP2 (Gamarnik et al. 2000) it is not surprising that translation efficiency is reduced when this ITAF is no longer able to bind efficiently.

Conclusion

miRNA-326 stimulates translation at the PV IRES. This effect can be diminished by mutating the binding site for miRNA-326 in the IRES. The mutant can be partially rescued by a complementary mutated miRNA. miRNA-127 and miRNA-422a had no significant effect on translation initiation at the PV IRES.

Mutation of miRNA-binding sites in the luciferase coding sequence

Negative controls (pD5) showed a severe inhibitory effect of miRNA-326* on firefly luciferase in RRL as well as when transfected into HeLa cells (figure 39 and 40).

Therefore binding sites for the seed region of miRNAs, 6 or 7 nts pairing, in the coding region of the firefly luciferase were identified by manual search using microsoft word. Neither miRNA-127mat nor its passenger strand miRNA-127* can bind in the firefly luciferase ORF, so miRNA-127 may serve as a negative control for unspecific inhibition by small double stranded RNAs.

As for miRNA-326, one binding site (6 nts) for the passenger strand miRNA-326* was identified, with an A at position 1 making it a 7mer-A1 site (Wu & Belasco 2005, Grimson et al. 2007). Additionally several 5 nt binding sites for miRNA-326* and miRNA-326mat (figure 51). The 6 nt binding site for miRNA-326* could be mutated without changing the amino acid composition of the firefly luciferase.

Several binding sites for miRNA-422a could be identified (figure 51), which cannot all be mutated silently so miRNA-422a was excluded from further experiments since there was no possible way to exclude an effect on the reporter.

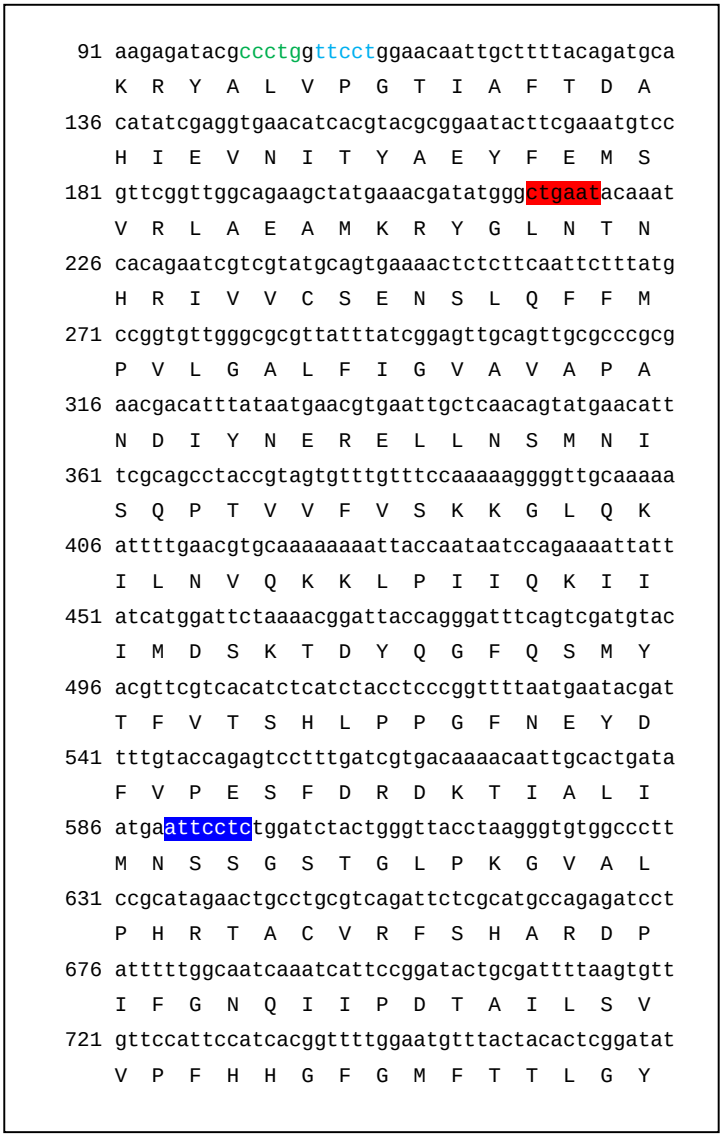

Figure 51: Part of the DNA sequence of firefly luciferase with translation into amino acid sequence. Potential binding site (5 nts) for miRNA-326mat is marked in green, the potential binding site (6 nts +A) for miRNA-326* is marked with a blue box, a further potential binding site (5 nts) for miRNA-326* is marked in blue, a potential binding site (6 nts) for miRNA-422a is marked with a red box.

The 6 nt binding site for miRNA-326* was mutated using site directed mutagenesis without changing the amino acid composition of the firefly luciferase reporter. The changed reporter was named silent mut326 to distinguish between the construct mutated in the IRES (as described above) and in the reporter. In vitro translation assays with the mutated reporter were performed in RRL (figure 52). The mutation does not have an effect on luciferase activity of the reporter.

Unfortunately the inhibiting effect of miRNA-326* was not diminished at high concentrations of miRNA, but rather enhanced. At low miRNA concentrations (25 ng and 50 ng) however no inhibition of translation efficiency at the mutated reporter can be observed (figure 52).

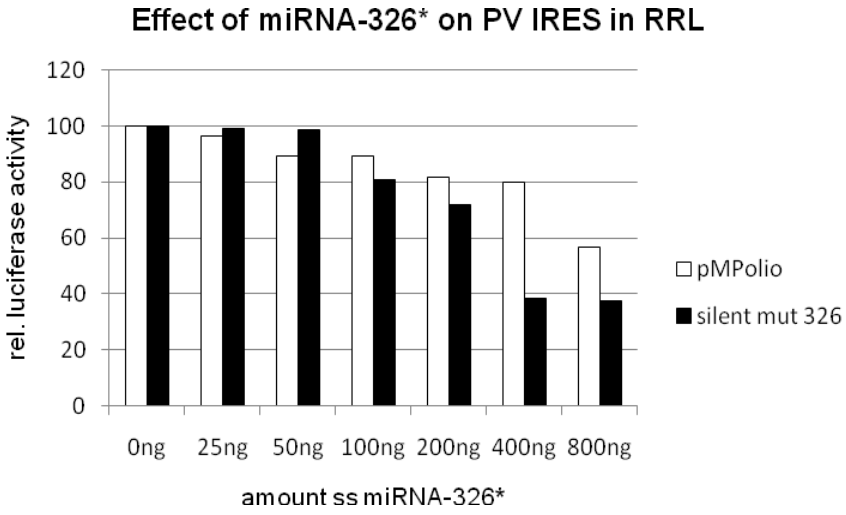

Figure 52: Influence of miRNA-326* on translation of the PV IRES luciferase mRNA with and without the silent mutation in RRL. All values are depicted in relation to reactions without addition of miRNA which is set to 100 %. Depicted values are the result of two independent experiments performed in duplicates therefore no standard deviation can be calculated.

Conclusion

There may be an effect of miRNA-326* on the firefly luciferase reporter coding region, however this effect cannot be diminished by mutating the binding site for miRNA-326* in the firefly luciferase coding region. The described results are therefore rather disheartening since an inhibitory effect of miRNA-326 on the reporter may mask a stronger stimulatory effect on the PV IRES.

Discussion

Purification of initiation factors and IRES trans-acting factors (ITAFs)

All initiation factors and ITAFs could be purified or expressed recombinantly. This took much longer than expected, due to unsuccessful attempts to establish a new purification protocol and long waiting periods for the starting material, rabbit reticulocyte lysate (RRL). To produce and ship the amounts necessary for purification of initiation factors, 3-6 months of processing time have to be taken into account. Taken all this together the unfortunate result is that I could not perform most of the experiments planned with the purified. However, a protocol for successful purification of eukaryotic initiation factors was established in the Niepmann Lab as described in methods and results (figure 1).

Initiation factors are conserved proteins in eukaryotes, and rabbit factors can substitute for human and even yeast factors without complications (Brown-Luedi et al. 1982). RRL is the most convenient starting material available and purification protocols from RRL are well established in several laboratories, e.g. Prof. Shatsky's lab.

In all protocols described in the literature about 30 – 40 % of initiation factors present in RRL are lost during purification (Safer et al. 1975). The total amount of proteins lost is constant, since it is due to irreversible absorption on beads and in void volumes of the columns. The percentage of loss increases when using less material, since net absorption does not change. Due to the loss it is important to start purification from sufficient material, at least three liters of RRL, in order to avoid loss of all initiation factors early during purification.

Using Prof. Shatsky's protocol (e.g. Pestova et al. 1996) I purified eIF3 without the p170 subunit (eIF3 (-p170), figure 6). eIF3 (-p170) still binds to the PV and HCV IRES and addition of eIF3 (-p170) to RRL allows IRES dependent translation (HCV, figure 23). Although it was shown that p170 is involved in binding to the 40S subunit, this function is shared with p110 (Hinnebusch 2006), which was still present in the preparation. Concluding from the fact that only deletion of both subunits (p110 and p170) in yeast proved to be lethal but not deletion of either one of them (Jivotovskaya et al. 2005), eIF3 seems to be functional even with degraded p170 as long as complete p110 is present. Fraser et al. (2004) showed binding of the eIF3 core (p170, p116, p110, p48, p47 and p40) and eIF3 p35 to the 40S subunit with degraded eIF3 p170, however they did not examine translation. P170 was shown to bind only

p116 directly (Methot et al. 1997, Fraser et al. 2004) and the other factors may be able to form a functional complex with or around degraded p170.

P170 in my eIF3 preparation may be functionally and structurally intact due to secondary and tertiary structure bounds (disulfide bounds, hydrogen bounds, hydrophobic or van-der-Waals interactions), which are destroyed when denaturing the protein for SDS-PAGE. In conclusion, it is unexpected but not impossible that eIF3 without p170 is still active in IRES dependent translation (Pulido et al. 2007) and it would be interesting to further investigate this phenomenon.

During the purification described in the methods and results eIF4G was also degraded leading to a loss of eIF4A and eIF4G on the Cap-Sepharose column (figure 5), since only the cap-binding moiety of eIF4F, eIF4E, was retained. Dissociation of eIF4F during purification on ion-exchangers has been observed before (Meyer et al. 1981, Wyckoff et al. 1990). Since it was planned to purify eIF4B from the flow through of the Cap-Sepharose column, a contamination of the flow through with eIF4A was rendering this approach impossible, since both proteins elute together from ion-exchange columns. As a consequence functional eIF4A (Nielsen et al. 1985, Yoder-Hill et al. 1993) and eIF4B were produced recombinantly.

In conclusion eIF3 and eIF4E could be obtained in highly pure form (Kemper et al. 1976) during my preparation and other factors obtained during prior purifications were given to me by Prof. Shatsky (complete eIF3 and eIF4F, as well as native eIF4B) or prepared recombinantly (eIF4A and eIF4B). These factors were used in UV-crosslinking experiments (figure 31) and to reconstitute the HCV IRES from purified components (figure 21).

UV-crosslinks

In order to investigate a possible synergistic effect of ITAFs binding to the PV IRES a short radioactively labeled RNA was constructed and incubated with pure protein preparations. Proteins bound to the RNA were irreversibly crosslinked by UV light. As shown in figure 34 the La autoantigen binds to the PV IRES to a much lesser extend when competing for binding sites with PTB or eIF4A.

Interaction of La with the PV IRES is dependent on the polypyrimidine tract (Haller et al. 1995) which is also recognized by PTB, indicating that both factors compete for the same binding site as described for the EMCV IRES by Kim & Jang (1999). PTB binding to the IRES remained unchanged when competing with La, while the band showing La binding to the IRES is much weaker than when La binds by itself. PTB may have a stronger affinity to the IRES than La and therefore out-competes La in a purified system with RNA as the limiting factor. However I did not have the time to try different concentrations of competitor PTB and

La, which would be useful, since PTB is known to inhibit translation initiation at the PV IRES at unphysiologically high concentration, which can be rescued by La (Kim & Jang 1999). This effect may be shown in crosslinks and further insights in the mechanism may be gained.
Competition with eIF4A totally abrogated La binding to the IRES, which may be explained by unwinding of secondary structures by eIF4A removing the synergy between stem loops (Haller et al. 1995) necessary for La binding. Since UV crosslinks as performed here do not freeze RNA-protein interactions in time, it is cannot be excluded that La binding to the PV IRES occurs before unwinding of secondary structures by eIF4A. Unwinding may displace La from the IRES. This could be further studied by addition of eIF4A after formation of the crosslink.
Taken together these results indicate a change of IRES structure by eIF4A during translation initiation (Martinez-Salas et al. 2010), which may influence binding of La and other ITAFs as well as initiation factors and ribosomal subunits.
eIF4A is necessary for translation initiation at the PV IRES, as shown by the use of a dominant negative mutant of eIF4A as an inhibitor of translation (Pause et al. 1994). Binding of eIF4A was shown here by UV-crosslink (figure 31), which concurs with the functional role for eIF4A in IRES dependent translation initiation.

Stimulation of translation initiation by PolyA tails

The poliovirus genome is naturally polyadenylated (Semler et al. 1984) and the polyA tail plays a role in infectivity of the virus (Sarnow 1989b). PolyA tails stimulate cap-dependent (Wells et al. 1998) and IRES-dependent (Bergamini et al. 2000, Thoma et al. 2004) translation. Naturally poliovirus has a polyA tail of 50-200 As (Ahlquist et al. 1979, Semler et al. 1984, Kavejian et al. 2004).
Cellular mRNAs are quasi-circular, due to interaction of the polyA binding protein (PABP) and the initiation factor eIF4G (Wells et al. 1998), but interactions between other 5' and 3' end binding proteins contribute to circularization as well (Jackson et al. 2010). During PV infection eIF4G is cleaved, thus the PABP binding part is no longer connected to the mRNA (Belsham & Sonenberg 2000), therefore circularization of PV mRNA must occur using a different mechanism.
There are two possible stages of translation which the polyA tail can influence:
Ribosome recycling: terminating ribosomes can easy re-initiate and commence a new round of translation due to close proximity of cap and termination signal and subsequent elevated local concentration of factors and ribosomal subunits (Mangus et al. 2003).

Initiation: Connection of both ends sends a signal to the 5' end that the mRNA is complete and may be translated into a complete polypeptide chain. This signal is achieved through conformational changes of eIF4G and eIF4E by binding to PABP (Borman et al. 2000).

My results indicate that polyA tails play an important role in initiation (figures 17 and 31) at the PV IRES. Translation initiation was stopped at the 48S stage or 80S stage (figures 17 and 31 respectively), elongation and termination did not take place, so the observed effect is solely on translation initiation. However an effect on termination and ribosome recycling cannot be examined with this experimental set-up. This additional effect is rather likely since translation efficiency was stimulated fourfold by addition of a $polyA_{50}$ tail (figure 16) while formation of 48S complexes was increased by only 30% (figure 17). The remaining stimulation may be due to an effect of polyA tails on elongation, termination or ribosome recycling.

Consistent with this the formation of 48S complexes at the EMCV IRES was not stimulated at all by addition of a 50A polyA tail, while several articles describe stimulation of translation under the control of the EMCV IRES by addition of polyA tails (Hruby & Roberts 1977, Bergamini et al. 2000). Possibly the stimulating effect on the EMCV IRES is solely on ribosome recycling rather than translation initiation.

To distinguish those two possibilities further experiments are necessary, for example separation of initiation complexes on sucrose gradients without inhibitors. This is rather difficult for translation initiation at the PV IRES since complexes form rather poorly (Ochs et al. 2002), but can be easily achieved for the EMCV IRES (Pestova et al. 1998).

Stimulation of formation of 48S complexes by addition of the polyA binding protein (PABP) to IRES dependent (Bradrick et al. 2007) and cap-dependent translation (Munroe et al. 1990, Kahvejian et al. 2005) was observed, indicating a role for PABP and subsequently polyA tails at the level of translation initiation. Excess of 40S subunits decrease the stimulating effect of polyA tails on capped mRNAs (Searfoss et al. 2001), which is another indication that polyA tails play a role in initiation under competitive conditions.

Even when using purified components (purified ribosomes, initiation factors and t-RNAs) on IRESs that do not need anything but ribosomes, like the CrPV IGN IRES, a lot of RNA (~50 %) remains outside of initiation complexes (Pause et al. 1994). When using polyadenylated mRNAs however more complexes are formed and subsequently RNA-Protein complexes without ribosomes decrease (figures 32 and 33). The effect of the polyA tail on translation on the PV IRES seems to be on the efficiency of complex formation and maybe displacement of endogenous inhibitors, which normally interfere with formation of 48S complexes and accumulate in the 20S RNP peak.

Identification of new ITAFs

Since the first reconstitutions of IRES elements from purified factors by Pestova et al. (1996 & 1998) several unsuccessful and unpublished attempts have been undertaken to reconstitute the prototypical IRES of poliovirus.

The first IRES which was reconstituted using only purified factors was the EMCV IRES (Pestova et al. 1996). Successful initiation was shown by toeprinting using 48S complexes stalled by GMP-PNP. Besides ribosomes and t-RNA, initiation factors eIF2, eIF3, eIF4B, eIF4F and PTB were the minimal requirement for initiation up to the formation of 48S complexes. The type III HCV and classical swine fever (CSFV) IRESs were reconstituted in 1998 (Pestova et al. 1998) and have even lower requirements: only ribosomes and eIF3 are sufficient to form 48S complexes, eIF2 needs to be added for subunit joining. Cap-dependent translation initiation has also been reconstituted from purified components (Dimitriev et al. 2003, Svitkin et al. 2009), requiring all known initiation factors but no additional proteins. However so far this approach was not successful with the PV IRES, since its requirements for ITAFs are very high and not all of them are known.

In order to identify new proteins, which might act as ITAFs for the PV IRES, 48S complexes were assembled using RRL supplemented with HeLa extract and several other compounds that enhance translation initiation, as described in the results section. The yield of 48S complexes was improved tremendously during my work, about 40 % of radioactively labeled RNA was incorporated into 48S complexes (figure 20), which is comparable to the 30 % incorporated at the EMCV IRES in Pestova et al. (1996) and is a vast improvement over previously published 48S complexes at the PV IRES (e.g. Ochs et al. 2002).

Isolation of proteins

The RNA used to form initiation complexes was internally labeled with biotinylated UTPs and can therefore be bound to streptavidin beads. Proteins bound to the IRES in 48S complexes are consequently retained on the beads as well. Proteins were eluted from beads using a combination of RNases and boiling the beads with SDS-PAGE sample buffer.

Similar approaches using biotinylated IRES RNAs bound to beads to identify ITAFs on picornavirus IRESs have been employed before (Blyn et al. 1995, Kim et al. 2004, Pacheco et al. 2008). However in those studies only parts of the IRES were used (Blyn et al. 1995) and all proteins bound to the IRES and beads (Pacheco et al. 2008) were investigated, not only those which are part of the 48S complex. Using biotinylated RNA Blyn et al. (1995) identified PCBP2 and two other proteins (40 and 42 kDa) that bind to stem loop IV of the PV IRES from RRL. Pacheco et al. (2008) identified several RNA binding proteins from cell extracts, by

coupling the FMDV IRES to Oligo-dt beads, but did not verify their results in functional assays. One study that verified its results by siRNA was Kim et al. (2004), who identified NSAP1 to bind to the IRES of HCV, a pestivirus, using biotinylated RNA.

In all described studies in vitro transcribed biotinylated RNA was bound to Streptavidin beads first and only then incubated with different lysates. Given the size of beads and the almost irreversible interaction between streptavidin and biotin this approach may result in the identification of any RNA binding proteins, but not necessarily in formation of initiation complexes on IRESs bound to Streptavidin beads. The identified proteins may not have an influence on IRES activity but just bind to it or to any RNA. A similar case was described for PTB (Back et al. 2002), which binds to the EMCV IRES but does not stimulate or inhibit its translation (Belsham & Sonenberg 1996). Since none of the studies used unrelated RNA, like the uncapped β-globin 5' UTR or a random RNA sequence of comparable length, identified proteins cannot be connected to translation initiation without doubt. Thus, in order to avoid identification of RNA binding proteins that are not part of the initiation complex, I allowed formation of 48S complexes on biotinylated RNA (figure 13) before incubation with Streptavidin beads. Separating complexes on sucrose gradients before binding to the beads ensures that proteins, which are part of the 48S initiation complex, can be purified separately from RNA binding proteins in the RNP complexes, which contain IRES-RNA complexes not functionally associated with ribosomes.

I did not expect to be able to detect initiation factors (figure 28) since they are known to dissociate from 48S complexes during sucrose gradient centrifugation (Jivotovskaya et al. 2005) especially on IRESs (Kolupaeva et al. 2005). However subunits of eIF3 can be clearly seen when separating complexes formed at the HCV IRES (figure 23).

Due to the low protein content of my elutions it was difficult to even identify known ITAFs on stained SDS-PAGE gels (figure 28). However I succeeded to identify a protein band of approximately 45 kDa that appeared in 48S complexes when assembled at the HCV IRES from extracts but not from purified components (figure 24). It appears to be present in 48S complexes assembled at the HCV and PV IRES (figure 28), but not in RNP complexes. Therefore this protein is most likely involved in formation of 48S complexes and translation initiation. The band at 45 kDa could also be seen clearly on coomassie stained SDS-PAGE gels (figure 25) when using translation reactions on the HCV and PV IRES without separation on sucrose gradients, since no RNA and proteins are lost due to the gradient and subsequent fractionation. The coomassie stained band was excised from the gel and analyzed using mass spectrometry.

Moreover another interesting band at about 50 kDa can be seen at the picornaviral IRESs EMCV and PV. It is not present with the HCV IRES (figure 25), indicating a protein specific for picornaviruses IRESs. This protein is present in RNP complexes but not in 48S complexes. It may be a picornavirus-specific inhibitor of translation. These bands were excised from the coomassie-stained SDS-PAGE gel (figure 28) as well and analyzed using mass spectrometry.

P45- cytoplasmic β-actin

The band seen at about 45 kDa at the PV and HCV IRES was identified as cytoplasmic β-actin, which is the monomeric form of β-actin-polymeres that form the actin tubules of the cytoskeleton. Since this protein is very abundant in lysates it is most likely an artifact of isolation and analysis. However association of viral translation and replication with parts of the cytoskeleton has been observed before (Pincheira et al. 2001). Picornaviruses are known to change the structure of the cytoskeleton (Rassmann et al. 2006) to construct an environment beneficial to their replication (Suhy et al. 2000). Although all described changes are due to interaction with tubulin but not actin, it is still possible that this interaction is physiological relevant. B-actin needs to be identified as an ITAF at least twice more before an artifact can be excluded with confidence.

P50- ErB3 binding protein (EbP1)

The picornavirus-specific band was identified as the ErB3 binding protein, EbP1, which is also known as $ITAF_{45}$ (Pilipenko et al. 2000). ErB3 is an epidermal receptor tyrosine kinase (Honda et al. 2007) and is regulated by EbP1 and 2, transcription factors probably involved in rRNA processing (Strausberg et al. 2001). EbP1 is known to shuttle between nucleus and cytoplasm (Squatrito et al. 2004), where it interacts with the ErbB3 receptor (Zhang et al. 2008). EbP1 was implicated in regulation of cap-dependent translation initiation via phosphorylation of eIF2. EbP1 or $ITAF_{45}$ is absolutely required for translation initiation at the FMDV IRES (Andreev et al. 2007). It was shown to enhance translation efficiency at the FMDV but not the EMCV IRES (Bonnal et al. 2005). Since EbP1 was detected in RNP-complexes but not in 48S complexes, it is possible that this protein binds to IRES structures (PV and EMCV), but does not have an effect on formation of 48S complexes, as described for PTB and the EMCV IRES before (Back et al. 2002). Functional assays using recombinant or native EbP1 in an in vitro translation system could help to clear this point.

Depletion

Another approach to gain further insight into the nature of the elusive ITAF was depletion of HeLa extract using PV IRES RNA. The depleted extract and beads were treated with the Ca^{2+} dependent micrococcal nuclease and the RNase inhibited by addition of EGTA (Pelham & Jackson 1976).

It was possible to deplete HeLa extract of its stimulatory effect on the PV IRES using this approach (figure 29), but the activity could not be restored by proteins eluted from the beads by RNase (figure 30). It is possible that the missing ITAF is not an RNA binding protein, but a protein interacting with an RNA binding protein, as described for SRp20 and PCBP2 by Bedard et al. (2007). These proteins could be lost during washing of the beads, however washes were not able to stimulate translation since known RNA-binding ITAFs, like PTB and La remain bound to the beads.

In conclusion, depletion did not reveal any novel ITAFs for the PV IRES or a new effect of combination of ITAFs. No novel reason for the tissue specificity of poliovirus could be identified. So, maybe the missing factor is not a protein at all, but something completely different, like a microRNA.

Effect of microRNAs on poliovirus translation initiation

MicroRNAs (miRNAs) play an important role in gene regulation in the cell (Pillai et al. 2007). Viruses infecting mammalian cells are also subject to regulation by miRNAs (Pedersen et al. 2007, Kelly et al. 2010). These miRNAs can act as inhibitors and enhancers of translation of viral mRNAs (Pedersen et al. 2007, Henke, Goergen et al. 2008). Since several miRNAs are expressed in a tissue specific manner (Langraf et al. 2007) it can be assumed that they play a role in defining the tissue type. Consequently I speculated if miRNAs may be a reason for the preferential translation of PV RNA in neuronal cells.

Identification of neuron-specific miRNAs

Several neuron-specific miRNAs were identified that potentially bind to the PV IRES using ViTa. The output of the program is an interactive picture depicting all miRNAs binding to the PV genome (Figure 35), the locations of the IRES (nt 1-nt 793), coding region (nt 794-nt 7372) and 3' UTR (nt 7373-nt 7440) were entered manually. It is noted that the first 130 nt of the PV 5' UTR are not strictly speaking part of the IRES (Nicholson et al. 1991), but form a cloverleaf structure essential for replication. Three miRNAs, miRNA-127, miRNA-326 and miRNA-422a were chosen for their binding sites in the PV IRES and their specificity for brain or neuronal tissue (figure 36).

Stimulation of translation by miRNA-326

Cell lines are a much more natural experimental set-up than the in vitro translation system RRL. I used the neuroblastoma cell line SH-SY5Y which has already been described as suitable for poliovirus translation and replication (LaMonica & Racaniello 1989), as well as HeLa cells.

miRNA-326 stimulated translation by the PV IRES in HeLa cells approximately two fold (figure 45). The stimulation of translation in the neuroblastoma cell line SH-SY5Y was about 1.6 fold (figure 46). This may be due to an internal factor in neuronal cells or endogenous miRNA-326 in SH-SY5Y cells and is consistent with the fact that the Sabin strains replicate much worse in SH-SY5Y than in HeLa cells (LaMonica & Racaniello 1989).

The majority of miRNA-effects described to today are inhibitory (Bartel 2009) and most miRNA targets are in unstructured regions of the 3' UTR of mRNAs (Lewis et al. 2005). The stimulation observed with miRNA-326 is therefore unusual in two ways: The binding site is in a structured region of the 5'UTR, and the effect is stimulating rather than inhibiting.

A few cases of stimulatory effects on translation by miRNA have been described (Vasudevan et al. 2007, Orom et al. 2008, Henke, Goergen et al. 2008). In two of them (Orom et al. 2008 and Henke, Goergen et al. 2008) the stimulating miRNAs (miRNA-10a and miRNA-122, respectively) bind to the 5' UTR of the mRNA for several ribosomal proteins (Orom et al. 2008) and the HCV polyprotein (Henke, Goergen et al. 2008). In contrast Vasudevan et al. (2007) describe binding of the miRNA let7 to the 3' UTR of mRNAs upon cell cycle arrest.

Vasudevan et al. (2007) showed the involvement of argonaut proteins (ago) and therefore the RNA induced silencing complex (RISC) in the stimulatory effect of let7 and miRNA-369-3p by western blot. On the other hand, Barnes et al. (2009) showed that RNAi against Ago2 in HeLa cells has no effect on PV replication and therefore translation. Both observations taken together may lead to the conclusion that the effect of miRNA-326 on PV differs mechanistically from the effect described by Vasudevan et al. (2007) and may not involve agos and RISC.

All described miRNAs as well as miRNA-326 decrease translation of some of their target mRNAs in a "normal" miRNA-way (Farh et al. 2005, Landgraf et al. 2007, Vasudevan et al. 2007, Orom et al. 2008). The stimulatory role seems to be an additional function only executed under special circumstances, like cell stress (Orom et al. 2008) and cell cycle arrest (Vasudevan et al. 2007). PV genomic RNA is not a natural target of miRNA-326 and this case might as well be hijacking of an established cellular mechanism by the virus for its own protein synthesis, similar to the use of ITAFs for translation initiation (Hellen & Sarnow 2001).

The binding site for miRNA-326 in the IRES was identified in a bulge in stem loop IV (figure 36). It was subsequently mutated from ACCCCA to ACGGGC. Care was taken not to destroy the bulge, which may be necessary for PV IRES activity (Ochs et al. 2002). The mutant was less active then the wild type (figure 50). Maybe the mutation had an effect on secondary structure and slightly altered IRES structure, despite all efforts to avoid this effect.. A mutation described by Gamarik et al. (2000) at the same position decreased binding of PCBP2 to the PV IRES.

Surprisingly a second binding site for miRNA-326 in the PV 5' UTR (table 2) also coincides with a binding site of PCBP2. This second binding site is part of the clover leaf structure necessary for replication but not translation of PV RNA (Gamarnik & Andino 1998, Gamarnik et al. 2000, see figure D1). miRNA-326 may interfere with PCBP2 binding to the IRES or the clover leaf or both. Direct binding of the seed sequence of miRNA-326 to the clover leaf is not likely, because the binding site is completely paired (figure D1).

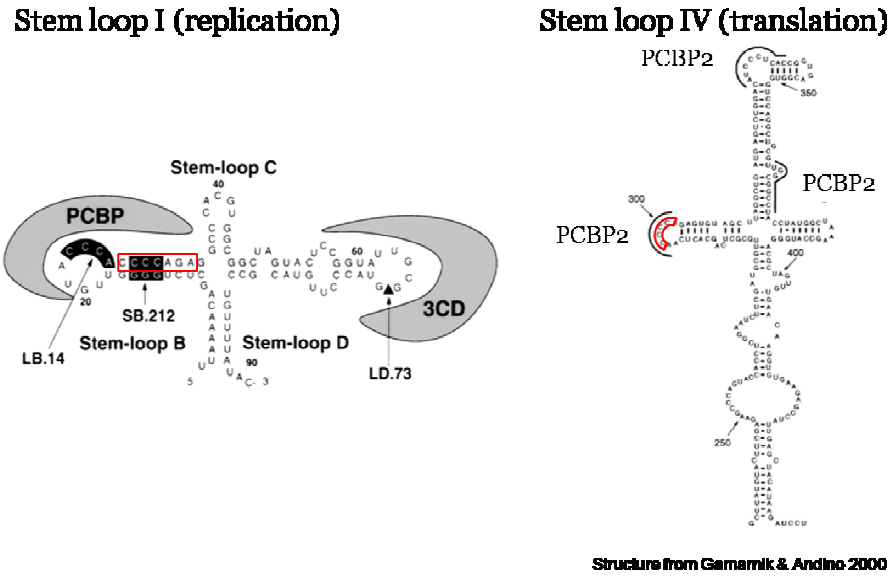

Figure D1: PV 5' UTR stem loop IV (IRES) and stem loop I clover leaf with miRNA-326 and PCBP2 binding sites. Stem loop IV from Gamarnik & Andino 2000, Stem loop I adapted from Parsley et al. 1997

Binding of PCBP2 to the IRES stimulates translation while binding of PCBP2 to the clover leaf enhances replication efficiency and inhibits translation (Gamarnik et al. 2000). PCBP2 consists of three KH-domains (Blyn et al. 1995), one of them (KH1) inhibits translation when binding to the PV 5' UTR by itself (Silvera et al. 1999). There are three regions in stem loop

IV of the PV IRES that are necessary for PCBP2 binding (Gamarnik et al. 2000), only one of them is covered by miRNA-326. This may impair PCBP2 binding to the IRES, but most likely does not totally abolish it. However, if this binding site coincides with the binding site of the inhibitory KH domain of PCBP2 (Silvera et al. 1999), masking of the binding site by miRNA-326 may have a stimulatory effect on translation initiation at the PV IRES.

This effect would be independent from the RISC or other normal miRNA-pathways. It could be tested experimentally by testing if the stimulation by miRNA-326 also occurs in PCBP2 depleted extract or with an IRES construct that does not contain the clover leaf and therefore the miRNA-326 binding site outside of the IRES. UV-crosslinking of PCBP2 and single PCBP2 KH domains to the IRES in the presence and absence of miRNA-326 is an easier method to verify an interaction of PCBP2, miRNA-326 and PV RNA.

Mutation of nts 299 to 302 in the PV IRES, removing the miRNA-326 binding site, also abolished the stimulatory effect by miRNA-326. This could be partially restored by a compensatory miRNA. If the effect was solely based on competition with PCBP2 binding, mutations in the mRNA and miRNA should not have an effect. Mutation of the same nucleotides by Gamarnik et al. (2000) impaired PCBP2 binding to the PV IRES as shown by RNA mobility shift assays. The mutated PV IRES was less active then the wildtype (figure 50), indicating an effect of impaired PCBP2 binding. Involvement of another miRNA-based mechanism is possible and can be tested for by western blots against ago proteins involved in formation of the RISC.

No effect on translation by miRNA-127 and 422a

miRNA-127 did not show any effect on translation controlled by the PV IRES. It is possible that this miRNA cannot reach its predicted target site, since the target site is paired as part of the stem of stemloop IV (figure 36). All other known target sites for miRNAs are in unstructured regions and secondary structure of RNA is thought to block miRNA-action (Bartel 2004, Grimson et al. 2007, Sun et al. 2009, Hauser et al. 2009).

The target site for miRNA-422a is also located in a bulge and unpaired. Co-transfection of miRNA-422a with the PV IRES reporter however showed a slight inhibitory effect in HeLa as well as in SH-SY5Y cells, but since it cannot be ruled out that this effect is on the reporter rather than on the IRES, no conclusions can be drawn.

Effect of miRNAs on PV IRES reporter constructs in RRL

The activity of ss miRNA in RRL was investigated. RRL is a highly promiscuous translation system, in which almost all cellular RNAs can be translated with high efficiency independent of cap or IRES structures (Pelham & Jackson 1976, Svitkin et al. 1996, Kayes et al. 2009).

However, viral RNAs, especially those under the control of picornaviral IRESs, do not translate well in RRL (Jang 2006). Important ITAFs are missing and need to be supplemented by addition of HeLa extract (Dorner et al. 1984) or purified components.
Several newer publications also indicate that RRL may not be a good system to study miRNAs since important proteins of the RISC seem to be present only in low abundance (Wang et al. 2006, Fabian et al. 2009). All results obtained in RRL are therefore to be examined with caution.

miRNA-binding site in luciferase

After the observation that miRNA-326* inhibits translation on the negative control pD5 (luciferase only) in RRL (figure 43 and 44), I identified several potential binding sites for miRNAs in the firefly luciferase (figure 51), but also in renilla luciferase and lux, a luciferase originating from the bacterium *Vibrio harveyi*. Most of the identified binding sites did not pair completely with the seed sequence of the miRNA but only with 5 or 6 pairing nucleotides. Only miRNA-326* had one site with full complementarity in the seed region.
Since neither miRNA-127mat nor its passenger strand miRNA-127* have a binding site in the firefly luciferase reporter, they can serve as controls for unspecific effects of short ss RNAs on the translation of the reporter. Neither of them inhibits the pD5 construct in RRL, an unspecific effect by ssRNA can therefore be ruled out.
For a miRNA to have an effect on mRNA at least 6 nucleotides have to pair in a Watson-Crick fashion (Ambros 2004, Lewis et al. 2005, Wu & Belasco 2005, Bartel 2009). 5 or even 4 pairing bases in active miRNAs have been observed, but require additional binding of the 3' UTR of the miRNA to the mRNA (Brennecke et al. 2005) which is not the case with the binding sites identified for miRNA-326mat and miRNA-326* in the luciferase reporter. I concluded that only one binding site for miRNA-326* is relevant for the effect on the reporter, which could be mutated without changing the amino acid sequence of the protein.
Most miRNAs investigated so far act on the 3' UTR of their target mRNAs. Nevertheless several sRNAs, the bacterial equivalent of miRNAs, act on the coding regions of their targets (Bouvier et al. 2008). Experimentally introduced miRNA binding sites in an extended version of the firefly luciferase were not able to inhibit translation (Gu et al. 2008), while miRNA binding sites introduced in the coding region of the PV polyprotein to achieve attenuation proved functional (Wilson & Richardson 2005, Barnes et al. 2008). However, Grimson et al. (2007) found single binding sites in the coding regions of mRNA to be unable to bind miRNAs using microarrays. Thus binding and effects of miRNA in ORFs of mRNAs seem to be an exception rather than the rule, but they can and do occur. Therefore it is possible that the full seed binding site for miRNA-326* have an effect on the luciferase reporter.

After silent mutation of the binding site for miRNA-326* in the firefly luciferase coding region the activity of the luciferase protein was not changed compared to the wildtype. However, the inhibiting effect of miRNA-326* in RRL was abrogated at low concentrations of miRNA-326*, but was even stronger at high concentrations (figure 52).
Maybe the incomplete binding sites (5 nts) are only targeted when a surplus of miRNA is present. To completely rule out this possibility all miRNA-326 binding sites in the luciferase have to be mutated, which is not possible without changing the amino acid composition of the reporter.

Conclusion

miRNA-326 has a stimulatory effect on translation on the PV IRES, which can be abrogated by mutating the binding site in the IRES. The other chosen miRNAs had no effect on translation via the PV IRES. The effect of miRNA-326* in the luciferase reporter could not be removed completely and might still interfere with the effect on the IRES. Several possible mechanisms of action by miRNA-326 have been speculated about above. Since miRNA-326 is developmentally regulated and poliomyelitis is a disease which mainly targets children, a role for miRNA-326 in tissue specificity and infection patterns is possible.

Conclusion and outlook

During my thesis I identified novel factors that influence translation initiation under the control of the Poliovirus IRES. This was done to further elucidate translation initiation on the prototypical IRES and gain further insight in the mechanisms of translation initiation in general. It was also done for the more practical reason to identify factors responsible for tissue tropism of poliovirus.
I optimized translation initiation at the PV IRES so that in vitro reconstitution improved tremendously. This was done in order to identify new protein factors which influence translation and lead to identification of EbP1, a known ITAF for the FMDV IRES. However, EbP1 might not be an enhancer, but rather an inhibitor of PV IRES dependent translation.
Additionally I identified a neuron-specific miRNA that stimulates translation by an unknown mechanism, which may not be a normal miRNA/RISC mechanism but part of an interaction between miRNA and ITAFs.
A more elaborate experimental setup including the PV 3'UTR (Dobrikova et al. 2003) and some viral proteins, like protease 2A and 3CD, could help to further elucidate potential interactions of PCBP2 and miRNA-326 as well as the effect of EbP1 on PV IRES dependent translation. Even though the 3' UTR seems to not have a strong effect on translation efficiency (Brown et al. 2004), it binds the known ITAF nucleolin, which stimulates translation

(Waggoner & Sarnow 1998), and “normal” miRNA interactions occur on the 3’UTR of mRNAs. Therefore use of a construct containing the 3’UTR of poliovirus would be helpful to gain insights into the mechanisms of translation initiation.

Effects of ITAFs and miRNA may be linked to each other as described by Makeyev et al. (2007) where translation of proteins in brain is upregulated by miRNA-124 through downregulation of PTB, which in turn inhibits alternative splicing.

The results obtained do not fully explain tissue tropism of poliovirus. However, I identified several novel regulatory factors involved in translation initiation at the PV IRES and maybe the effect of miRNA-326 on translation at the PV IRES is a small step on the way to explain the tissue tropism of poliovirus. This opened a lot of new and exciting questions which remain to be answered in the future.

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Appendix

Plasmid maps

Maps of plasmids used to generate templates for in vitro transcription of mRNA, which were then used for transfection and in vitro translation (figure 1 and 2). Figure 3 shows the plasmid used to generate RNA for sucrose gradient centrifugation. This system uses a pGL3 backbone which has different 5'UTRs inserted before a firefly luciferase reporter.

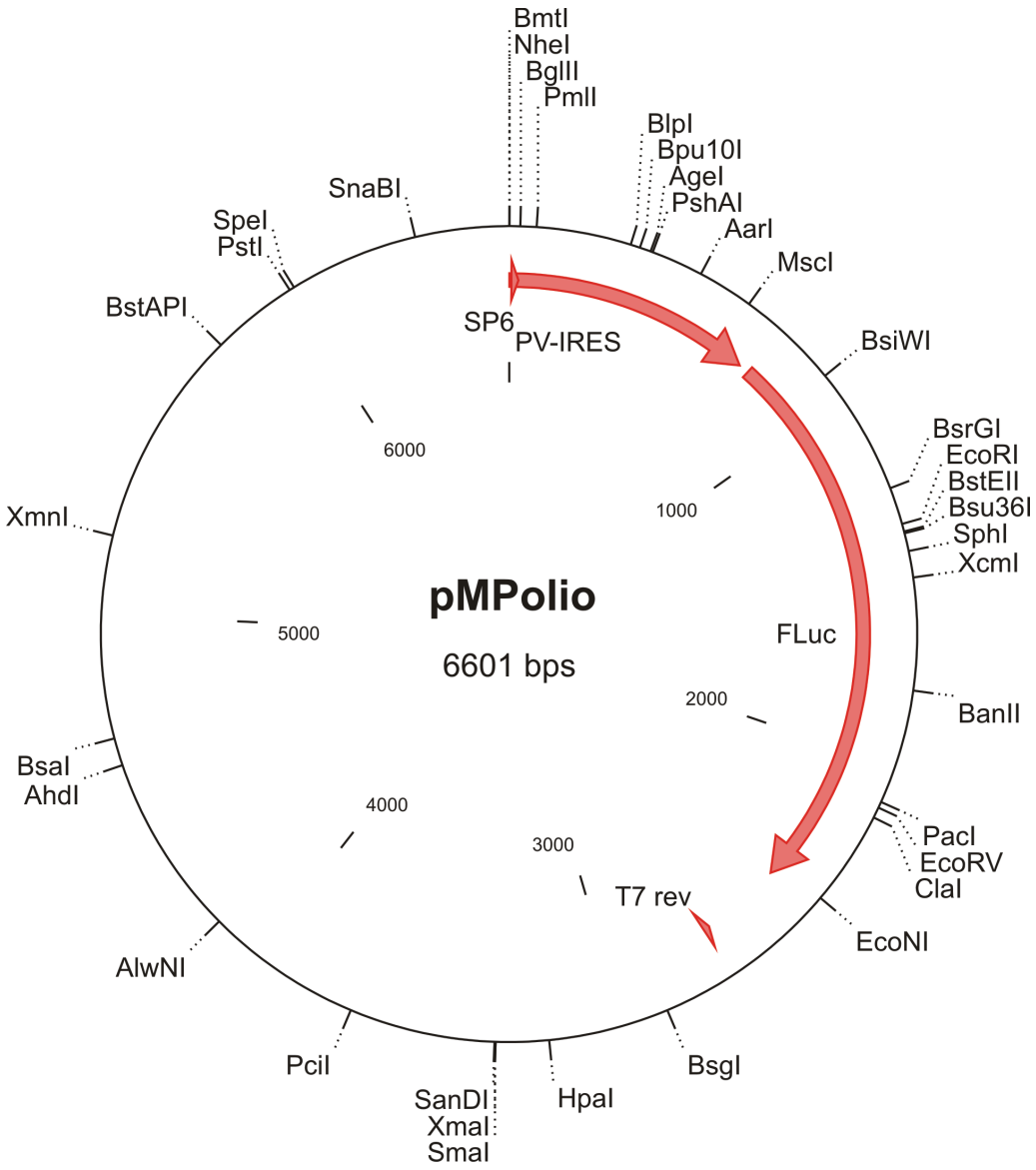

Figure 1: Plasmid pMPolio, used to generate template for RNA in vitro transcription by digestion with SmaI.

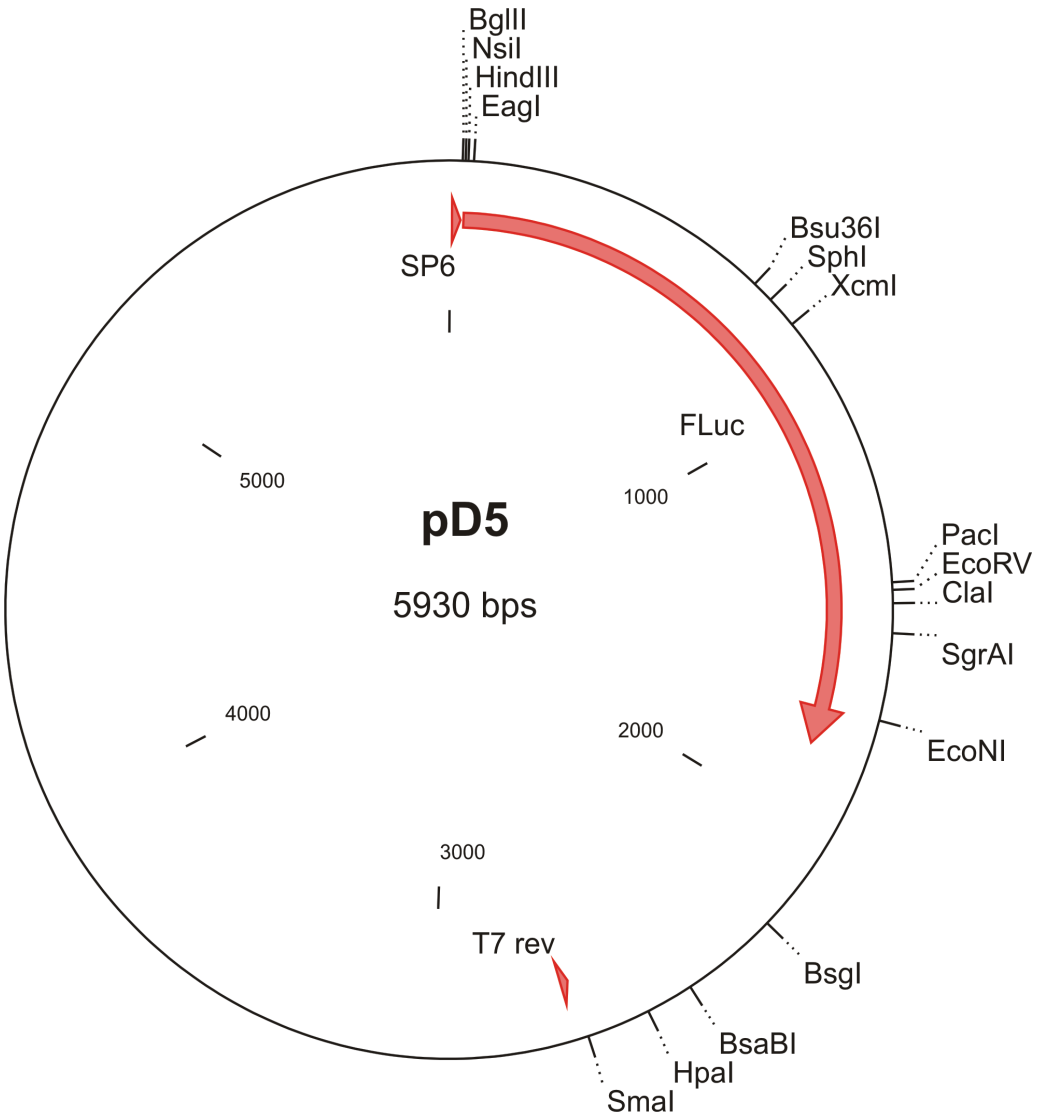

Figure 2: Plasmid pD5, used to generate templates for in vitro transcription of firefly luciferase control construct by digestion with SmaI

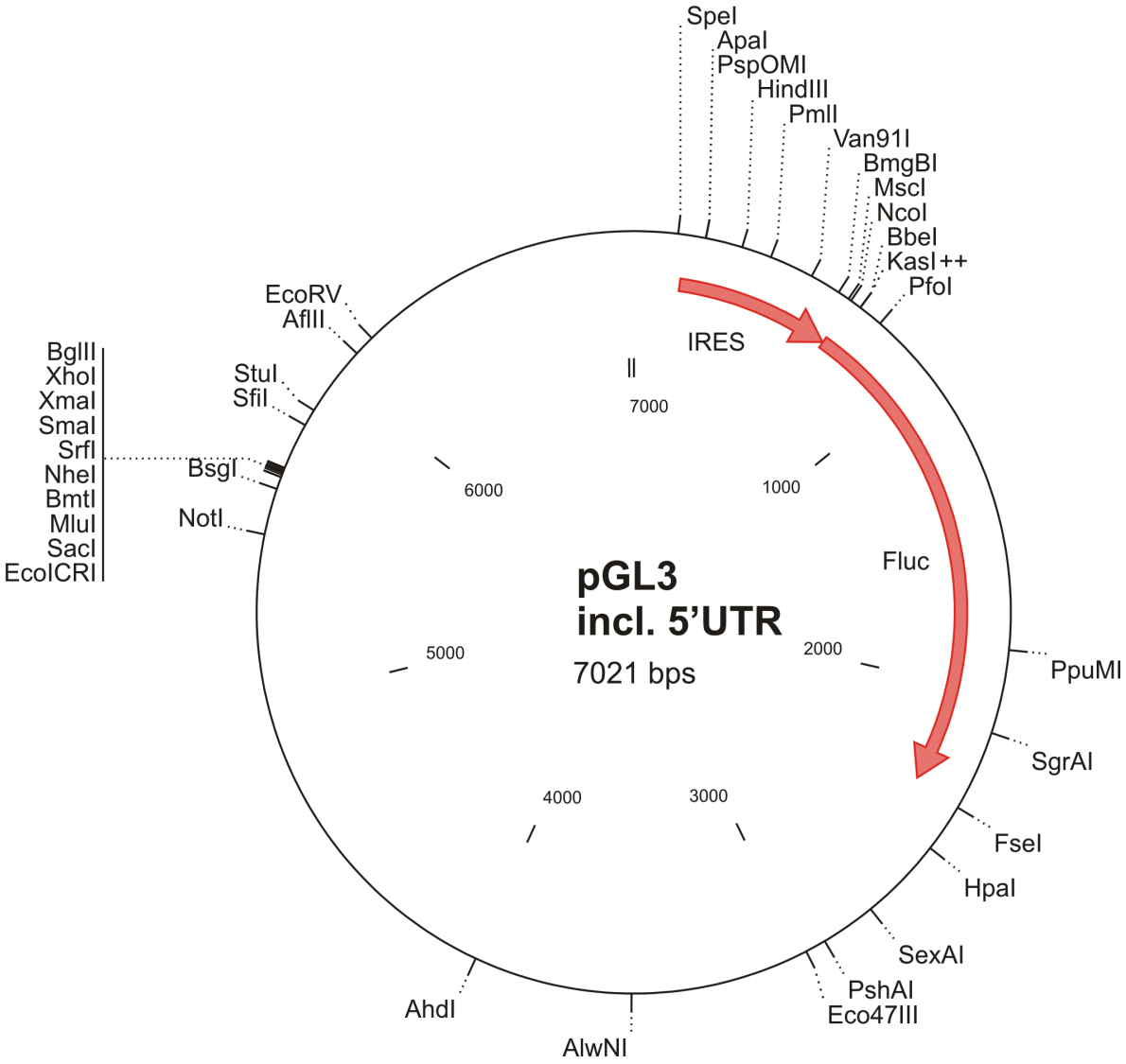

Figure 3: Plasmid pGL3 used with IRESs of PV, EMCV, HCV or the 5' UTR of beta-actin

Ammonium sulfate precipitation

of ammonium sulfate	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

Adapted from "Data for Biochemical Research" (R.M.C. Dawson, D.C. Elliott, and K.M. Jones, eds.), 2nd Ed. Oxford Univ. Press, London, 1969.

Northern blots against miRNA-326 in cells

To identify endogenous miRNA-326 northern blots were performed using a DIG labeled RNA probe complementary to full length miRNA-326 mat. Total small RNA was isolated from HeLa and SH-SY5Y cells and transferred to a nylon membrane with semi-dry electroblotting. To control for RNA quality and normalise different amounts of RNA between cell lines, U6 snRNA (Lim et al. 2003) was detected using a DIG-labeled U6 probe (figure 53).

No miRNA-326 could be detected in either cell line owing either to the low sensitivity of the technique or the fact that the neuron specific miRNA-326 is not expressed in either cell line.

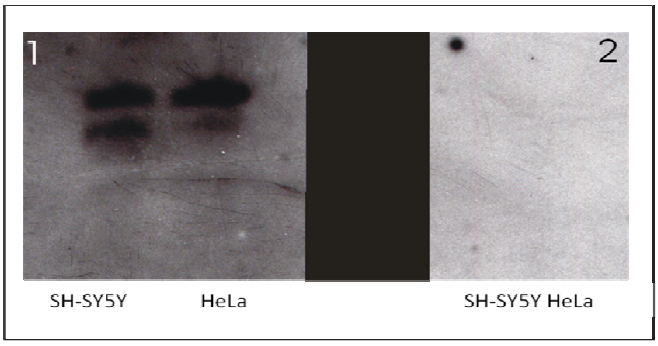

Figure 53: Northern blot, detecting miRNA-326 (2) and U6 snRNA (1) in total small RNA isolated from 10^6 SH-SY5Y and HeLa cells with DIG-labeled probes.

Discussion

Northern blots performed using a DIG labeled probe against miRNA-326 mat failed to detect endogenous miRNA-in SH-SY5Y or HeLa cells (see appendix).

miRNA-326 is expressed in a developmentally regulated manner, especially abundant during neuronal growth (Landgraf et al. 2007). SH-SY5Y however are differentiated cells, so miRNA-326 may not be expressed in high amounts here.

Newborn transgenic mice are especially susceptible for PV infection (Kauder & Rancaniello 2004) maybe because in their developing brain miRNA-326 is upregulated. The paralytic effects of PV infection occur mainly in children (Colbere-Garapin et al. 1989), this may be due to the development of neurons and coupled to the expression of miRNA-326. This can be explained by the fact that infection of differentiated neuronal cells with poliovirus always lead to cell lysis while a percentage of undifferentiated cells are able to sustain a persistent infection. Rapid cell lysis leads to clearance of infection before permanent damage can be done to the nervous system (Benton et al. 1996).

Eidesstattliche Erklärung

Ich erkläre, die vorgelegte Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt zu haben. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, sind als solche gekennzeichnet. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis eingehalten.

Gießen, Mai 2010

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