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DIPLOMARBEIT

Titel der Diplomarbeit

„Proof-of-principle for the *in vivo* StreptoTag method-based isolation of RNA binding proteins“

Verfasser

Stephan Glanz

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1. Summary

RNA-protein interactions play a key role in fundamental cellular processes in living organisms. As this type of interaction is a common regulator in pathogenic networks, it is of special interest to be investigated. A number of *in vitro* methods have been established to isolate RNA binding proteins. However, these *in vitro* approaches may result in artificial interactions that would not occur within cellular conditions. Here, the “StreptoTag” method for isolation of RNA binding proteins *in vivo* is presented. The StreptoTag method, a novel technique to identify RNA-protein interactions, employs *in vitro* transcribed RNA target sequences, which are tagged with an RNA aptamer (STag) specifically binding to streptomycin. RNA binding proteins from crude cell extracts may bind to the chromatographically immobilised target RNA and are then co-eluted by addition of streptomycin. In the presented work, the system was adapted for *in vivo* applications. Therefore, putative protein target STag-RNA was transiently expressed in eukaryotic cells, allowing RNA-protein interactions to occur under physiological conditions. For a proof-of-principle, we isolated the human immunodeficiency virus-1 (HIV-1) RNA binding protein Rev from HEK293 cells by expressing STag-RNA harbouring the Rev responsive element (RRE). Standardisation of the method included high-level target RNA synthesis in transfected cells and optimisation of binding conditions, allowing high affinity interaction of Rev with the STag-RRE-RNA. In the final step, target RNA and Rev protein were co-expressed in HEK293 cells and successfully purified by affinity chromatography. In conclusion, the established *in vivo* StreptoTag method may facilitate isolation and identification of currently unknown RNA binding proteins and further protein interacting factors in cellular environments.

2. Zusammenfassung

RNA-Protein Interaktionen spielen eine Schlüsselrolle in fundamentalen zellulären Prozessen aller lebenden Organismen. Diese Art der Interaktion ist vor allem in pathogenen Netzwerken involviert und folglich von besonderem Interesse. Eine Vielzahl *in vitro* basierender Methoden für die Isolierung RNA bindender Proteine wurde etabliert, wobei diese jedoch oftmals zur Isolierung von Artefakten führen, welche unter zellulären Bedingungen nicht anzutreffen wären. Die hier vorgestellte neuartige Methode ist eine Technik zur Identifizierung von RNA-Protein Interaktionen basierend auf einem *in vitro* transkribierten RNA Aptamer (STag) und dessen spezifischer Bindung an Streptomycin. RNA-bindende Proteine aus rohem Zellextrakt binden im Folgenden an die chromatographisch immobilisierte Ziel-RNA und können durch Zugabe von Streptomycin co-eluiert werden. In der vorliegenden Arbeit wurde dieses System für eine Anwendung *in vivo* angepasst. Hierzu wurden eine STag-RNA mit einer Protein-bindenden Sequenz transient in eukaryonten Zellen exprimiert, und damit die RNA-Protein Interaktion unter zellulären Bedingungen ermöglicht. Zum Nachweis, dass dieses Prinzip grundsätzlich funktioniert, wurde das HIV-1 RNA-bindende Protein Rev aus HEK293 transfizierten Zellen isoliert. Für die Standardisierung der Methode war ein hohes Syntheselevel der Ziel RNA in transfizierten Zellen sowie die Optimierung der Bindungskonditionen von Rev zur SRS-RNA ausschlaggebend. Im finalen Schritt wurden HEK293 Zellen mit dem RNA- sowie dem Protein-kodierenden Vektor co-transfiziert und das Rev Protein erfolgreich durch Affinitätschromatographie aufgereinigt. Die hiermit etablierte *in vivo* StreptoTag Methode sollte zukünftig die Isolierung sowie Identifizierung von bislang unbekannten RNA-bindenden Proteinen sowie Protein-interagierenden Faktoren in zellulärem Umfeld erleichtern.

3. Introduction

3.1 RNA - protein interactions

RNA-protein interactions play a key role in fundamental processes of all living organisms. For example, interaction of the Epstein-Barr virus (EBV)-encoded RNA EBER-1 and the host cellular ribosomal protein L22 correlates with growth-promoting properties (Houmani, Davis et al. 2009). Similarly, the breast cancer susceptibility gene BRCA1 is posttranscriptionally regulated by the RNA binding protein HuR (Saunus, French et al. 2008). Furthermore, RNA-protein interactions are of central importance in host-pathogen interactions as for example in human immunodeficiency virus (HIV)-1, where multiple RNA-interacting proteins mediate reverse transcription of RNA and integration of cDNA into the host genome. So far, mainly *in vitro* methods were available for identifiction of RNA-protein interactions, e.g., those which are based on the interaction of biotin with streptavidin. This method implies biotinylation of target RNAs which are subsequently immobilised on streptavidin-coupled sepharose or magnetic beads. Cell extracts are mixed with these beads and target proteins interacting with the biotinylated RNA are retained upon elution. As this method is based on posttranscriptional modification of RNA *in vitro*, non-physiological conditions may contribute to formation of protein-RNA interactions that would not have occured under physiological conditions within the cell. Moreover, since the secondary and tertiary structure of the target RNA determines successful protein binding (Zhang, Jun Cho et al. 2010), posttranslational modification such as biotinylation might impair RNA structure and therefore contribute to a decreased specificity of such *in vitro* based assays. Previously, the StreptoTag method, a new chromatography-based purification technique for RNA-binding proteins, has been developed (Bachler, Schroeder et al. 1999) and was further improved by alteration of the StreptoTag RNA aptamer to increase specificity (Dangerfield, Windbichler et al. 2006).

3.2 The StreptoTag method

3.2.1 The basics of streptomycin-RNA interaction

Streptomycin is a member of the aminoglycoside antibiotics. Its specific interaction with RNA was identified by interfering with ribosomal subunits. The antibiotic has been shown to suppress missense and nonsense codons *in vivo*, and to induce misreading of the genetic code *in vitro* (Davies, Gilbert et al. 1964; Pestka, Marshall et al. 1965; Garvin, Biswas et al. 1974). Specific interaction of streptomycin with ribosomal RNA was shown by chemical modification assays performed with ribosomes bound to streptomycin (Moazed and Noller 1987). Furthermore, streptomycin was found to interact with, and thus inhibit self-splicing of group I intron RNA (von Ahsen and Schroeder 1991). *In vitro* selection, applying affinity chromatography and chemical probing, using dimethylsulfate and kethoxal, revealed streptomycin-binding RNA aptamers and defined secondary structures. The minimal size of an aptamer required to bind to streptomycin was determined to be a 46-mer RNA (see Figure 3.2.1.1). The presence of Mg^{2+} was found to be a prerequisite to facilitate binding of streptomycin to RNA (Wallace and Schroeder 1998).

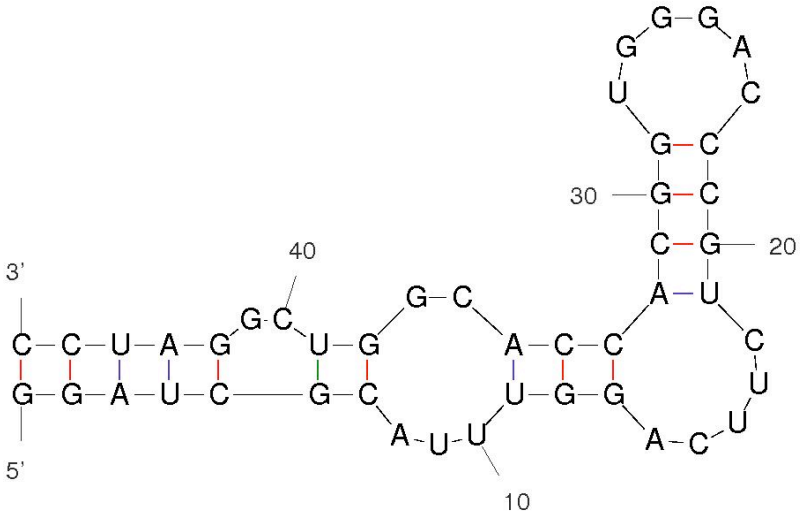


Figure 3.2.1.1 Streptomycin-binding RNA aptamer. A 46-mer RNA aptamer was determined to be the prerequisite for streptomycin binding to RNA (virtual folding carried out using the "mfold" webserver; Zuker, 2003).

3.2.2 The StreptoTag method and its modifications

For further application, i.e. the isolation of RNA-binding proteins, the streptomycin-binding RNA aptamer (StreptoTag) was expressed as a hybrid RNA fused to the putative protein binding RNA sequence of interest (Figure 3.2.2.1). The Strepto-tagged hybrid RNA was then loaded onto a dihydrostreptomycin-coupled sepharose column; the subsequently applied RNA-binding protein is retained on the column and eluted upon addition of streptomycin (Bachler, Schroeder et al. 1999), Figure 3.2.2.2). In order to increase affinity and specificity of the StreptoTag aptamer, the original AGGGU apex of the hairpin, nucleotides 24 to 28, was modified to form a GCAAG loop (Figure 3.2.2.3), resulting in enhanced stability and increased binding efficacy to streptomycin from 65.3 % to 81.3 % (Dangerfield, Windbichler et al. 2006). Furthermore, larger RNA molecules of up to at least 550 nt were capable to efficiently bind to the streptomycin-coupled column material, thus making this enhanced GCAAG aptamer the tag of choice for the work presented hereafter.

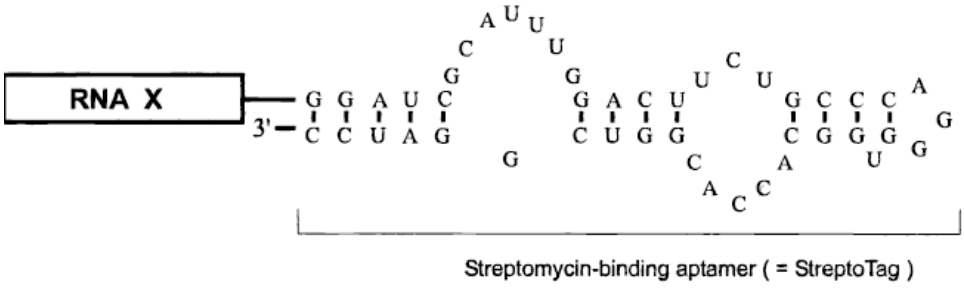


Figure 3.2.2.1 Hybrid Strepto-tagged target RNA. The streptomycin-binding RNA aptamer (StreptoTag) is linked to the putative protein binding RNA (RNA X) at the 5' end and expressed as a hybrid (Bachler, Schroeder et al. 1999).

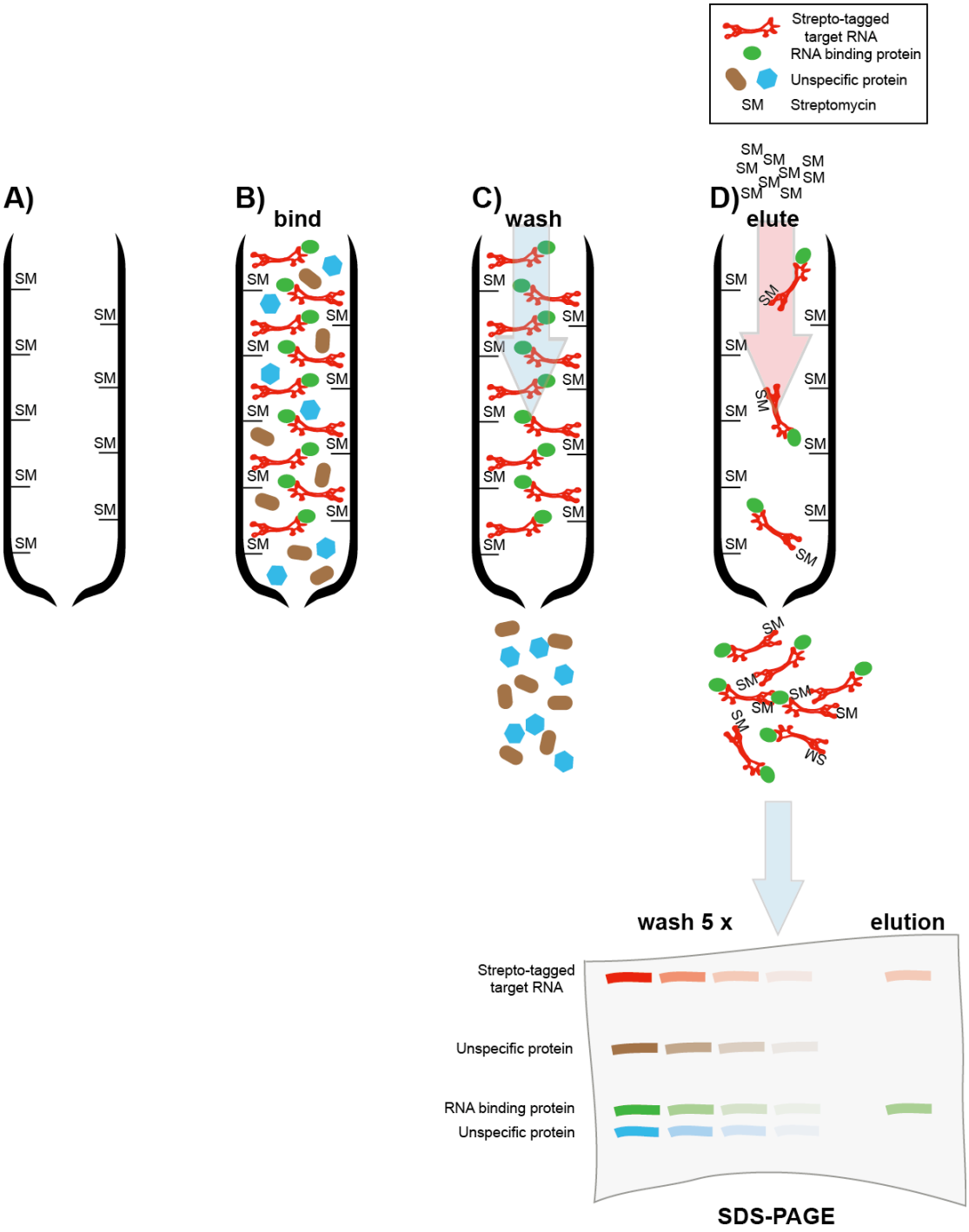


Figure 3.2.2.2 Schematic drawing of the StreptoTag-based affinity purification. A) The sepharose column material is coupled with dihydrostreptomycin. **B)** RNA-binding proteins are directly applied to the column. **C)** The RNA-bound protein is retained on the column due to the interaction of the StreptoTag and the streptomycin-coupled sepharose. Unspecific cellular proteins are removed in 4-5 consecutive washing steps. **D)** The RNA-bound protein of interest is eluted upon addition of streptomycin, separated by gel electrophoresis and detected using Western blotting or silver staining and mass spectrometrical analysis.

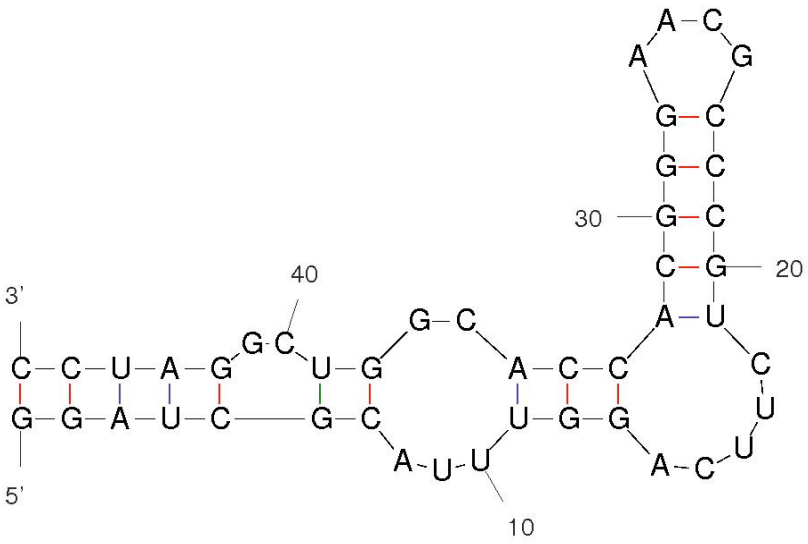


Figure 3.2.2.3 Altered streptomycin-binding RNA aptamer. Modification of the nucleotides 24 to 28 of the apex of the StreptoTag hairpin from AGGGU to a GCAAG tetraloop resulted in increased stability and binding efficiency (Dangerfield, Windbichler et al. 2006); virtual folding carried out using the “mfold” webserver; Zuker, 2003).

3.3 Viral RNA-protein interactions

3.3.1 HIV-1 regulator of virion expression (Rev) and Rev responsive element (RRE)

For establishment of the StreptoTag method-based affinity purification of proteins *in vivo*, a well investigated system was chosen. Therefore, the well known (Freed 2001) RNA-protein interaction of the HIV-1 regulator of virion expression (Rev) protein and the Rev responsive element (RRE) RNA was used as a model. The HI-virus is an RNA retrovirus with a complex gene regulation.

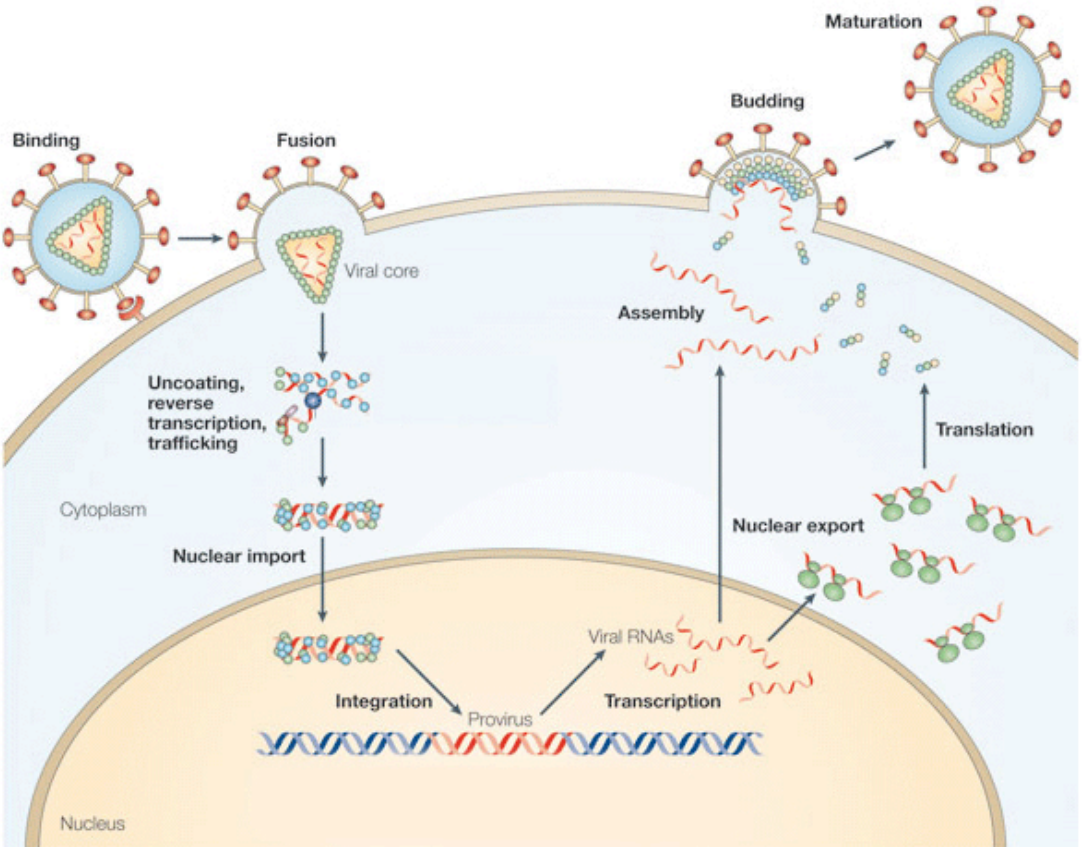


Figure 3.3.1.1 Retroviral replication cycle. Following binding and fusion of the viral particle, retroviral RNA is reversely transcribed into cDNA and integrated into the host genome. Viral RNAs are transcribed from provirus DNA and translated into viral proteins, which assemble in the cytoplasm to form a new functional retrovirus (adapted from Nisole, Stoye et al. 2005)).

In the retroviral life cycle (Figure 3.3.1.1), after fusion with the host cell, viral RNA is reversely transcribed into cDNA. The cDNA is subsequently integrated into the host genome. Utilizing the hosts transcription and translation machinery, provirus DNA is transcribed and spliced as well as mRNAs translated into the regulatory proteins Tat, Nev and Rev. On the other hand, the Rev protein binds to unspliced viral RNAs carrying the RRE, such as RNAs encoding the Gag, Pol, Env, Vif and Vpr proteins, thereby mediating their export from the nucleus to the cytoplasm. The viral proteins assemble in the cytoplasm and a new functional viral particle is budding from the cellular surface, (Figure 3.3.1.1, adapted from Nisole, Stoye et al. 2005).

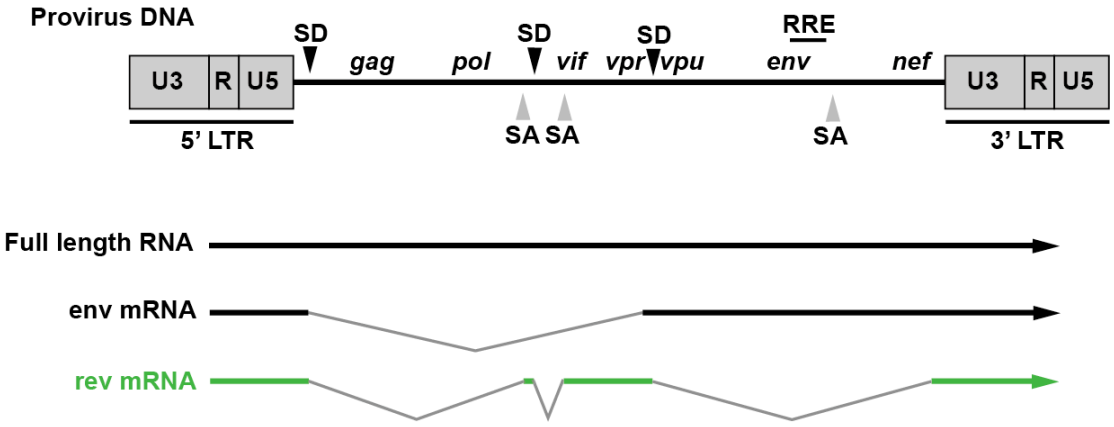


Figure 3.3.1.2 HIV-1 provirus DNA and splicing of full length RNA into subgenomic mRNA. LTR, Long terminal repeats; **U3**, Unique region 3; **R**, Repeat region; **U5**, Unique Region 5; **SD**, Splice Donor; **SA**, Splice Acceptor; **RRE**, Rev responsive element; **gag**, group-specific antigen; **pol**, reverse transcriptase, integrase, protease; **vif**, viral infectivity factor; **vpr**, viral protein R; **vpu**, viral protein U; **env**, envelope; **nef**, negative regulatory factor;

Transcription initiating at the HIV-1 5' LTR generates a large number of viral RNAs. These are classified in 1) unspliced RNAs, which function as mRNAs for the Gag and GagPol polyprotein precursors, 2) partially spliced mRNAs, which encode the Env, Vif, Vpu and Vpr proteins and 3) multiply spliced mRNAs, which are translated into Rev, Tat, and Nef (Purcell and Martin 1993, Figure 3.3.1.2). As most cellular mRNAs are transported out of the nucleus fully spliced, the need for unspliced and partially spliced RNAs in the cytoplasm poses a problem for HIV. This has been overcome through the evolution of the viral protein Rev (Regulator of virion expression) and a cis-

acting RNA element, the RRE (Rev responsive element), carried by those unspliced and partially spliced RNAs to be exported (Figure 3.3.1.3).

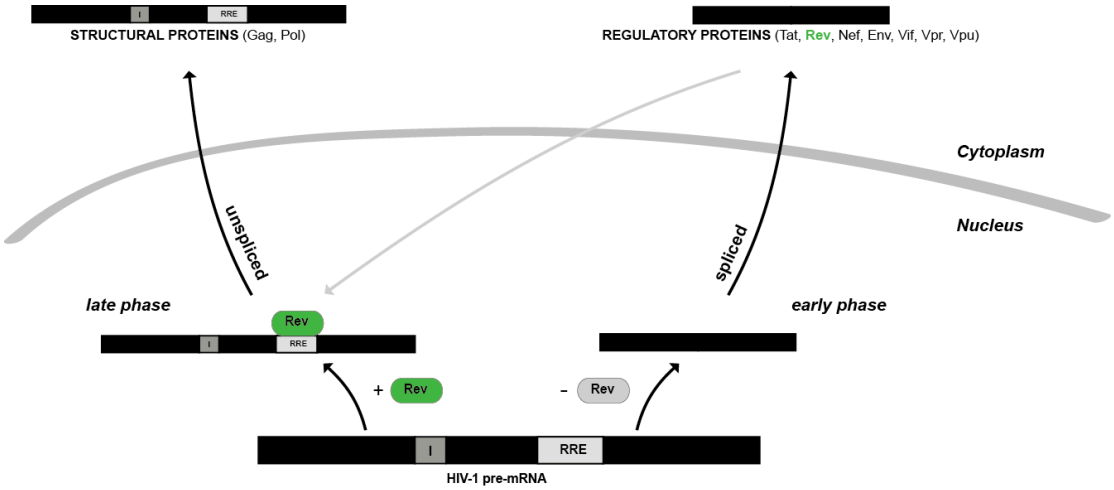


Figure 3.3.1.3 Export of mRNA in HIV-1. HIV-1 export of spliced RNA and Rev-dependent export of unspliced and partially spliced RNA in the late phase of the viral replication cycle. **I**, intron.

Rev is a 13 kDa phosphoprotein composed of 116 amino acids which are encoded by two exons, both of which are essential for protein function (Pollard and Malim 1998). The determined molecular weight of Rev protein is 17 kDa, consistent with other proteins rich in basic amino acid residues (Nalin, Purcell et al. 1990). Rev contains two functional domains: an arginin-rich sequence required for RNA binding and nuclear localisation, and a hydrophobic leucine-rich motif that mediates nuclear export. The 250 nt long Rev responsive element (RRE) is located in the *env* gene and is present in all unspliced and partially spliced HIV-1 RNAs (Freed 2001). The HIV-1 Rev protein multimerises into eight molecules by binding to the RRE, thereby forming a complex capable of interacting with the cellular nuclear export machinery. Consequently, the RRE-containing unspliced or partially spliced RNA is transported to the cytoplasm. Rev then shuttles back to the nucleus, using its nuclear localisation signal (NLS) (Rosen, Terwilliger et al. 1988; Maldarelli, Martin et al. 1991; Figure 3.3.1.3).

3.3.2 MMTV regulator of export of MMTV mRNA (Rem) and Rem responsive element (RmRE)

Similarly to the well established HIV-1 Rev/RRE RNA-transport system, an equivalent but less investigated interaction has been recently described for the mouse mammary tumor virus (MMTV). This virus belongs to the family of complex retroviruses. It encodes regulatory as well as accessory proteins such as a superantigen (Sag) (Acha-Orbea and Palmer 1991; Marrack, Kushnir et al. 1991), a negative-acting factor (Naf) (Salmons, Erfle et al. 1990) and a regulator of export of MMTV mRNA (Rem), (Indik, Günzburg et al. 2005). Analogue to the HIV-1 Rev protein, the MMTV Rem is required for nuclear export of unspliced viral RNA, which is carrying the Rem responsive element (RmRE). The RmRE is a 490 nt long region that spans the env-3'LTR junction (Müllner, Salmons et al. 2008; Figure 3.3.2.1).

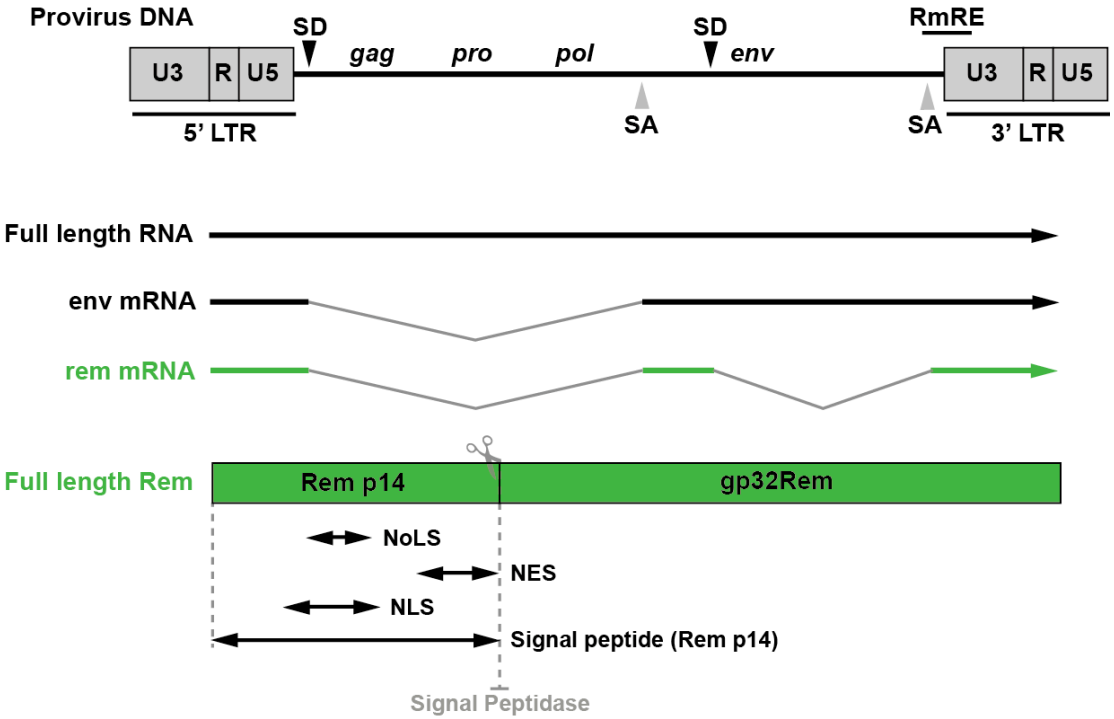


Figure 3.3.2.1 MMTV mRNA splicing and generation of Rem p14 by cleavage of full length Rem (adapted from Byun, Halani et al. 2010)**. LTR**, Long terminal repeats; **U3**, Unique region 3; **R**, Repeat region; **U5**, Unique Region 5; **SD**, Splice Donor; **SA**, Splice Acceptor; **RmRE**, Rem responsive element; **NoLS**, Nucleolar localisation sequence; **NES**, Nuclear export sequence; **NLS**, Nuclear localisation sequence.

The MMTV Rem protein has a calculated molecular weight of 34 kDa and is a product of the doubly spliced 906 nt long pre-mRNA transcript. Due to two potential asparagine-linked glycosylation sites, which in case of glycosylation increase the molecular weight by 2.5 kDa each, the actually observed molecular weight of Rem is 39 kDa. The protein contains a highly basic arginine-rich N-terminal region which resembles a NLS (nuclear localisation signal) and confers to RNA binding abilities as well as to nuclear localisation of the protein. A leucine-rich domain, which in HIV-1 Rev is referred to as the NES (nuclear export signal), is also present in MMTV Rem. For nuclear function, Rem protein requires proteolytic processing by a signal peptidase at the endoplasmic reticulum. Subsequent, the Rem(p14) signal peptide re-translocates into the nucleus to mediate export of unspliced viral RNA (Figure 3.3.2.2).

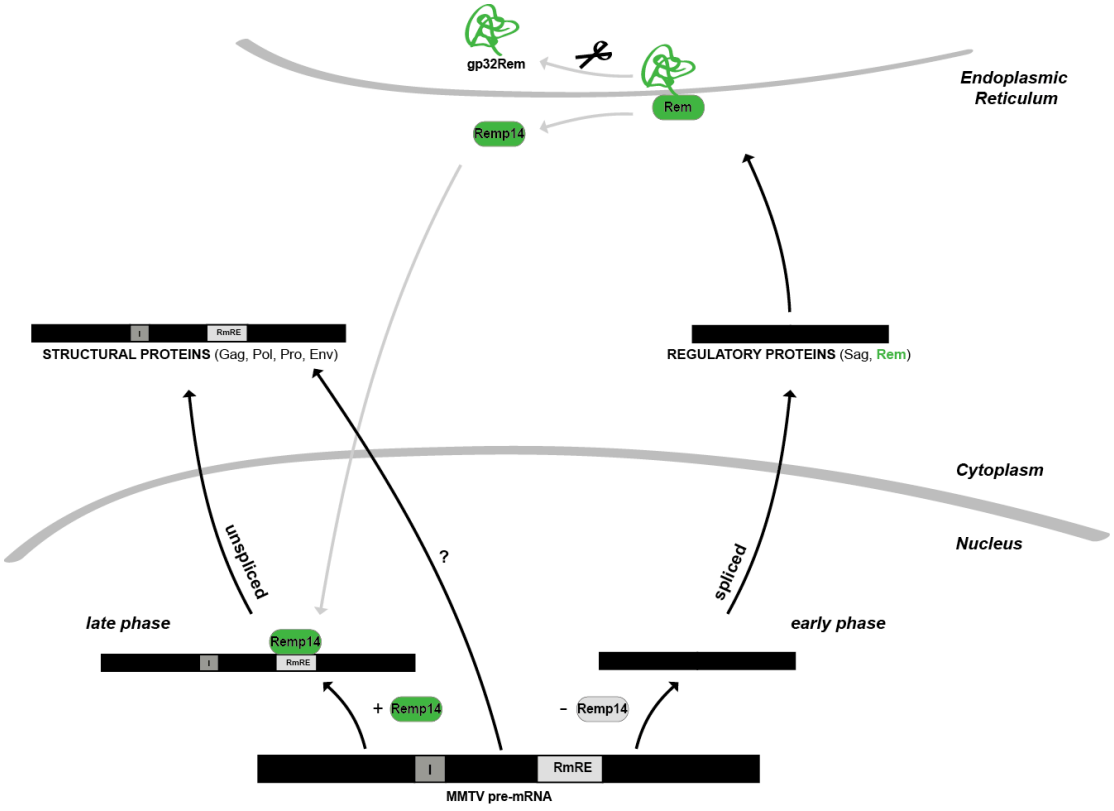


Figure 3.3.2.2 Export of mRNA in MMTV infected cells. MMTV export of spliced RNA and Rem-dependent export of unspliced RNA in the late phase of the viral replication. The nuclear export of single-spliced env mRNA is accomplished via a yet unknown distinct, Rem-independent export mechanism (Müllner, Salmons et al. 2008). Full-length MMTV Rem undergoes proteolytic cleavage in the ER (endoplasmic reticulum) resulting in gp32Rem and Rem(p14) which is transported back into the nucleus. **I**, intron.

Since unspliced RNA is essential but its export is prevented in the retroviral life-cycle, retroviruses evolved mechanisms to circumvent the cellular splicing machinery. In MMTV infected cells the export of unspliced viral RNA is accomplished by the Rem/RmRE system. The presence of the NLS and NES consensus sequences suggests that MMTV Rem shuttles between the nucleus and the cytoplasm (Indik, Günzburg et al. 2005), thereby exporting RRE-carrying unspliced viral RNA from the nucleus into the cytoplasm. As HIV-1 Rev, also MMTV Rem protein multimerises during binding to the RmRE to become functionally active (Mertz, Lozano et al. 2009). The 490 nt long RmRE spans the Env-LTR junction region and its presence is mandatory for the Rem-dependent export of the unspliced viral RNA encoding the proteins Pol, Pro, and Env. In contrast, single spliced env mRNA is exported from the nucleus via a yet unknown, distinct, Rem-independent export mechanism (Müllner, Salmons et al. 2008).

3.3.3 StreptoTag-mediated Rev-RRE binding *in vitro*

For establishment of the StreptoTag method *in vivo*, a stepwise approach was chosen in order to avoid complications and allow modifications and optimization of each single step. The work presented hereafter, is a continuous follow up of previous achievements such as enhancement of the StreptoTag RNA aptamer (Dangerfield, Windbichler et al. 2006). Recent approaches (Müllner Matthias, Diploma Thesis 2007) have shown binding of Strepto-tagged RNA to dihydrostreptomycin-coupled sepharose columns as well as an increase in binding affinity of a double Strepto-tagged RNA aptamer in comparison to the use of a single StreptoTag. Finally, recombinantly expressed HIV-1 Rev was purified using *in vitro* transcribed double Strepto-tagged HIV-1 RRE RNA (SRS) (Müllner Matthias, Diploma Thesis 2007). Based on these findings, the present study should start by reproducing StreptoTag-mediated affinity purification of recombinantly produced HIV-1 Rev protein and *in vitro* transcribed SRS-RNA.
Thereafter, in two different steps, *in vitro* components should be substituted to allow investigation of RNA-protein interaction in an *in vivo* setting. The system

should be established based on the well-known interaction of HIV-1 Rev and its responsive RNA element, the RRE.

3.3.4 Aim of this project

The aim of this project was to adapt and establish the StreptoTag method for application under physiological conditions. Therefore, a stepwise approach from affinity purification of *in vitro* transcribed RNA and FPLC-purified recombinant protein towards a purification of protein-RNA complexes from co-transfected cells was chosen. The stepwise approach was designed as follows :

1. Affinity purification using *in vitro* transcribed double Strepto-tagged HIV-1 RRE (SRS) and FPLC-purified recombinant HIV-1 Rev
2. Affinity purification of cellular HIV-1 Rev using *in vitro* transcribed SRS-RNA
3. Affinity purification of cellular HIV-1 Rev directly from HEK293 cells co-transfected with the SRS and Rev encoding vector.

Within the first step in establishment of the method it should be determined if double Strepto-tagged RRE is able to bind to and be retained by the dihydrostreptomycin-coupled sepharose column and allows recognition and binding to the secondary structure by Rev. Second, it should be investigated if the purified recombinant HIV-1 Rev is functional and thus able to bind to its responsive element in order to be purified.
After successful isolation of recombinant HIV-1 Rev using *in vitro* transcribed SRS-RNA, the second step should be to isolate the protein directly out of HEK293 cells transiently transfected with the HIV-1 Rev encoding vector. As this is supposed to be an intermediate step, still *in vitro* transcribed SRS-RNA should be used for affinity purification of cellular Rev. A successful detection of cellular HIV-1 Rev either by Western blot or silver staining will be pre-requisite to proceed, as sufficient amounts of protein have to be produced for proper detection. Furthermore, in advance to the final step, the cellular

expression level of SRS-RNA should be determined to ensure that the minimum level of SRS-RNA, which is required for the successful isolation of HIV-1 Rev in the first step, is transcribed.

In the final step, the StreptoTag method should be applied in a completely physiological environment. Therefore, HEK293 cells will be transiently co-transfected with the HIV-1 Rev as well as the SRS encoding vector allowing the RNA-protein interaction to occur under cellular conditions. Cell lysates will be prepared without disrupting protein interactions and directly applied to the column. Transiently expressed cellular HIV-1 Rev bound to *in vivo* transcribed SRS-RNA should be retained on the column due to the affinity of the StreptoTag to the dihydrostreptomycin-coupled sepharose column. Upon elution and separation in an agarose gel, the protein should be detectable either by Western blotting or silver stain.

4. Materials and Methods

4.1 Recombinant DNA technology

4.1.1 Virtual cloning

Cloning of plasmid DNA, restriction endonuclease digests as well as design of DNA oligonucleotide primers for polymerase chain reaction (PCR) were planned using the Clone Manager 5 from Sci-Ed software. Using Clone Manager 5 it was possible to predict sizes of cloning products, annealing temperatures of primers applied in PCR (see materials and methods 4.7), sizes of PCR products as well as calculating DNA fragment sizes resulting from restriction endonuclease digests (see materials and methods 4.1.2) in fully annotated plasmid maps.

4.1.2 Restriction endonuclease digests

Each restriction endonuclease digest was simulated before, using the software Clone Manager 5 (see materials and methods 4.1.1). The program generated a list of all DNA fragments that should be obtained by electrophoretical separation.
All restriction enzymes were purchased from Promega and used with the included corresponding buffer as well as bovine serum albumine (BSA). When performing a double digest, the most suitable buffer for both enzymes was chosen as recommended by the manufacturer. Restriction endonuclease digests were performed in 1.5 ml polypropylene tubes (Eppendorf) in a volume of 20 µl with a maximum of 1 µg DNA per 10 units of enzyme.
After components were mixed properly by pipetting, the mixture was incubated at 37°C for 1 hour. For preparative digests, the amounts were scaled up linearly to a maximum of 500 µl reaction volume.

Reaction mixture for a standard analytical digest :

1 µg plasmid DNA
2 µl 10x buffer
2 µl BSA (1 µg/µl)
1 µl restriction enzyme (10 u/µl)

ddH_2O up to 20 µl

4.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis is the standard method to separate DNA fragments by size. By applying an electric current, negatively charged nucleic acids migrate through the agarose gel to the cathode in a gel of a percentage of 0.5%-2%, corresponding to the expected size of the fragment to be analysed. Smaller fragments are less retained in movement and therefore migrate faster than larger ones. Gels were prepared by mixing agarose (Invitrogen) in 1 x TAE buffer and followed by cooking in a microwave oven at 750 W until the agarose was dissolved completely. The gel was left on the magnetic stirrer for 10 min to cool down below 50°C and warm agar was poured into the prepared gel chamber holding a comb (PEQLab Biotechnology). After 25 min, the gel was cured and the chamber filled with 1 x TAE buffer. Samples with 5 x loading buffer were loaded into the slots and separated by application of an electric current of 90 V and 500 mA for 1-2 hours, depending on the size of the fragments. Following separation, the gel was stained in a 1 x TAE buffer containing 0.5 µg/ml ethidium bromide (Sigma). Ethidium bromide is absorbed by the gel, incorporates into DNA and shows fluorescence upon exposure to UV-light of 254 nm in wave length, making separated DNA fragments visible. For documentation, the gel was photographed using the UV Vilber-Lourmat gel documentation system.

50xTAE buffer :	2 M	Tris base
	7.51%	Acetic acid (v/v)
	50 mM	Ethylenediaminetetraacetic acid (EDTA)
	pH 8.0	

5xLoading buffer :	0.25%	Bromphenol blue
	0.25%	Xylene cyanol
	0.25%	Orange G
	30%	Glycerol
	10 mM	EDTA
	in ddH_2O	

4.1.4 Purification of DNA fragments from agarose gels

For further processing, the DNA fragment of interest was excised using a clean scalpel under UV-light (UV-lamp Vilber-Lourmat) with the lowest intensity possible to avoid radiation-induced damage of DNA. For DNA purification, the Wizard SV Gel and PCR Clean-Up System from Promega was used. According to the protocol, membrane binding solution at a ratio of 10 µl per 10 mg of agarose gel slice was added to a 1.5 ml polypropylene tube and incubated at 65°C in a heating block until the agarose was dissolved completely. Next, the mixture was transferred to the SV minicolumn and incubated 1 min at room temperature following centrifugation at 16000 x g for 1 min (Eppendorf table top centrifuge). The flow-through was discarded and the column washed by adding 700 µl wash solution and centrifugation at 16000 x g for 1 min. Again the flow-through was discarded and the washing step repeated with 500 µl of membrane wash solution and centrifugation at 16000 x g for 5 min. The collection tube was emptied and centrifuged for 1 min with the lid open to allow evaporation of residual ethanol. The SV minicolumn was placed in a clean 1.5 ml polypropylene tube and to elute DNA bound to the resin, 50 µl of ddH_2O were added, incubated for 1 min at room temperature and then spun down for 1 min at 16000 x g. The concentration of

purified DNA was determined (see materials and methods 4.1.5) and either stored at -20°C or directly used.

4.1.5 Determination of nucleic acid concentrations

DNA as well as RNA concentration measurements were performed with a spectrophotometer GeneQuantPro from GE Healthcare. Therefore, samples were diluted 1 : 40 and then exposed to UV-light of 260 nm wavelength. The more nucleic acids the solution contained, the higher the absorption of UV-light and the resulting concentration calculated. The DNA to protein ratio measured at 260 nm and 280 nm, respectively, should range from 1.6 to 1.9, indicating limited protein contamination.

4.1.6 Processing of 5’ and 3’ DNA ends

In order to produce recombinant plasmid DNA, different fragments either with blunt or sticky ends need to be joined. Therefore it is necessary to process the 3’ and 5’ ends, respectively, in order to proceed. If the ends of DNA fragments were cut with different restriction enzymes and are incompatible, a blunt end ligation is considered. To generate blunt ends, T4 DNA polymerase was used for removing 3’ overhangs mediated by its 3’ - 5’ exonuclease activity, and filling 5’ overhangs mediated by 3’ - 5’ polymerase activity. To avoid intra-molecular self-ligation of DNA fragments, terminal phosphate groups were removed by dephosphorylation using alkaline phosphatase derived from calf intestine (CIAP - Promega).

T4 DNA polymerase mixture :	20 µl	5 x T4 DNA polymerase buffer
	20 µl	0.5 mM dNTP mix (Promega)
	0.5 - 0.25 µg	DNA
	2 µl	T4 DNA polymerase (5 U/µl)
	in ddH_2O up to 100 µl final volume	

The reagents were mixed by pipetting followed by incubation at 11°C for 15 min. Subsequently the DNA was purified using the Wizard SV Gel and PCR Clean-Up System (see materials and methods 4.1.4).

Dephosphorylation :	5 µl	10 x dephosphorylation buffer
	1 µl	CIAP enzyme (0.1 U/µl)
	1 – 25 µg	DNA
	in ddH_2O up to 50 µl final volume	

After proper mixing, incubation followed at 37°C. After 30 min an additional 1 µl of CIAP was added and incubated for further 30 min. CIAP was inactivated by incubation at 56°C for 30 min. Dephosphorylated DNA was purified using the Wizard SV Gel and PCR Clean-Up System (see materials and methods 4.1.4).

4.1.7 DNA Ligation

To join either sticky or blunt ended DNA fragments, T4 DNA ligase was used to catalyse formation of phosphodiester bonds between the 5' phosphate end of one and the 3' hydroxyl end of another nucleotide. The vector and insert fragments were ligated at a molar ratio of 1 : 3, with concentrations determined by a spectrophotometer (see materials and methods 4.1.5).

Conditions for sticky ends :	4 µl	5 x ligase reaction buffer
	0.01-0.1 µg	total DNA
	0.1 unit	T4 DNA ligase
	in ddH_2O up to 20 µl final volume	
	23°C	incubation temperature
	1 h	incubation time

Conditions for blunt ends :	4 µl	5 x ligase reaction buffer
	0.1-1 µg	total DNA
	1 unit	T4 DNA ligase
	in ddH_2O up to 20 µl final volume	
	14°C	incubation temperature
	16-24 h	incubation time

Following ligation, DNA was precipitated with butanol to minimize salt concentrations that might impair efficiency of subsequent bacterial transformations. Therefore the reaction volume was increased to 50 µl with ddH_2O and 500 µl of 1-butanol were added. After vortexing for 30 sec, the precipitate was spun down by centrifugation for 30 min. at 16000 x g. Supernatant was discarded carefully and the DNA containing pellet dried in an Eppendorf Speed-Vac vacuum dryer followed by resuspension in 20 µl of ddH_2O.

4.1.8 Transformation of plasmid DNA into E.coli

For amplification of generated recombinant plasmids, electrocompetent bacteria (Electromax electrocompetent DH10B bacteria, Invitrogen) were transformed by electroporation. Transformation is achieved by applying an electric current of 1.8 kV that causes poration of the cell membrane and thus allows a very efficient uptake of foreign DNA.
Precipitated ligated DNA (see materials and methods 4.1.7) was mixed with 25 µl of freshly thawed electrocompetent bacteria and incubated on ice for 5 min. The mixture was pipeted into a 1 mm pre-cooled electroporation cuvette (Biozym) and immediately electroporated at 1.8 kV (BioRad Pulse Controller Plus) followed by a quick uptake in 1 ml of 37°C pre-warmed SOC medium (Invitrogen). Transformed bacteria were incubated for 1 hour at 37°C on a shaker. Subsequently 100 µl of the suspension were plated on an agar plate containing suitable antibiotics for proper selection of successfully transformed bacteria and further incubated at 37°C over night.

4.1.9 Bacterial culture media and agar plates

Growth media and agar plates contained L-Broth medium (LB) as a main component and were prepared in 5 l batches.

LB medium :	1%	NaCl
	1%	Select peptone 140
	0.5%	Yeast extract
	pH 7.5 using 10 M NaOH	

Agar plates :	15 g	Bacterial grade agar (Difco)
	1 l	LB medium
	antibiotic for selection (for example ampicillin 100 mg/ml)	

Bacterial grade agar was dissolved in LB medium, autoclaved and allowed to cool down to 50°C, then the heat sensitive antibiotic was added. The mixture was poured into bacterial culture dishes with 10 cm in diameter and after hardening of the agar, plates were stored at 4°C for mid-term use.

4.1.10 Plasmid extraction in small scale (Miniprep)

After transformation, plasmids had to be isolated out of bacterial clones and checked for integrity. Therefore, a single colony was picked from the agar plate and inoculated in 3 ml LB-medium containing the corresponding antibiotic and incubated at 37°C under constant shaking over night. The next day, 1.5 ml of the bacterial culture were harvested by centrifugation at 4000 x g for 5 min. The bacterial pellet was resuspended in 300 µl resuspension buffer P1 and incubated at room temperature for 5 min. 300 µl of lysis buffer P2 were added, the tube mixed by inverting and incubated at room temperature for 5 min following addition of 300 µl neutralisation buffer P3. The lysate was centrifuged at 16000 x g for 30 min and plasmid containing supernatant was transferred to a new 1.5 ml polypropylene tube. Addition of 650 µl isopropanol led to precipitation of DNA which was spun down by

centrifugation at 16000 x g for 30 min. The supernatant was discarded and the DNA-containing pellet washed in 500 µl 70% ethanol. The DNA pellet was spun down again, the supernatant discarded and the plasmid DNA-containing pellet resuspended in 50 µl ddH_2O. DNA concentration was determined (see materials and methods 4.1.5) and restriction endonuclease test digests were carried out (see materials and methods 4.1.2).

Resuspension buffer P1 :	50 mM	Tris-HCl pH 8.0
	10 mM	EDTA
	100 mg/ml	RNase A
Lysis buffer P2 :	200 mM	NaOH
	1%	SDS (w/v)
Neutralisation buffer P3 :	3 M	KCl
	pH 5.5	adjust with glacial acetic acid

4.1.11 Plasmid extraction in large scale (Maxiprep)

When plasmids were found to be correct after the test digest, a large scale plasmid extraction was performed using the Qiagen Plasmid Maxi Kit. Therefore the 1.5 ml left over of miniprep over night culture was used to inoculate 200 ml of LB medium containing the appropriate antibiotic. The 200 ml culture was incubated in a 2 l Erlenmeyer flask under vigorous shaking over night at 37°C. If necessary, 5 ml of the culture were taken and frozen as a bacterial stock at -20°C or -80°C for further processing. The bacteria were harvested by centrifugation at 6000 x g for 15 min. After discarding the supernatant, the bacterial pellet was resuspended in 10 ml of resuspension buffer P1 (Qiagen). After 5 min, 10 ml of lysis buffer P2 (Qiagen) were added, the sealed tube inverted several times and incubated for exactly 5 min at room temperature. For neutralisation, 10 ml of neutralisation buffer P3 (Qiagen) were added and the lysate incubated on ice for 20 min. The bacterial debris was spun down first at 20000 x g for 30 min and a second time at 20000 x g

for 15 min, both at 4°C and subsequently the plasmid-containing supernatant was applied to a QIAGENtip 500, previously equilibrated with 10 ml equilibration buffer QBT (Qiagen). The column was washed twice with 30 ml of washing buffer QC (Qiagen) and finally bound plasmid DNA was eluted with 15 ml of elution buffer QF (Qiagen). For precipitation of eluted plasmid DNA, 10.5 ml of isopropanol were added and precipitated DNA collected by centrifugation at 4°C with 15000 x g for 30 min. Supernatant was discarded and the pellet washed with 5 ml of 70% EtOH. Again the pellet was spun down at 15000 x g for 10 min at 4°C and the supernatant decanted. The plasmid DNA-containing pellet was air dried in an Eppendorf Speed-Vac vacuum dryer and resuspended in 200 µl ddH_2O. DNA concentration was measured (see materials and methods 4.1.5) and restriction endonuclease test digests were carried out (see materials and methods 4.1.2).

Buffer P1, P2, P3 :	see materials and methods 4.1.10	
Equilibration buffer QBT :	750 mM	NaCl
	50 mM	3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.0
	15%	Isopropanol (v/v)
	0.15%	Triton X-100 (v/v)
Wash buffer QC :	1 M	NaCl
	50 mM	MOPS pH 7.0
	15%	Isopropanol (v/v)
Elution buffer QF :	1.25 M	NaCl
	50 mM	Tris-HCl pH 8.5
	15%	Isopropanol (v/v)

4.1.12 Long term storage of bacteria

For long term storage 1.2 ml of the bacterial over night culture were mixed with 200 µl of 80% glycerol and then stored at -80°C.

4.1.13 Isolation of genomic DNA from eukaryotic cells

For isolation of genomic DNA from cultured cells, the DNeasy Blood & Tissue Kit (Qiagen) was used. A maximum of $5x10^6$ cells were harvested by centrifugation at 300 x g for 5 min. The pellet was resuspended in 200 µl of PBS and then 20 µl of proteinase K (800 U/ml, Sigma) were added. After addition of 200 µl buffer AL (Qiagen), cells were mixed by vortexing and incubated at 56°C for 10 min. 200 µl of absolute ethanol were added and the sample vortexed again. The mixture was pipetted into a DNeasy Mini spin column (Qiagen) and centrifuged at 6000 x g for 1 min. Flow-through was discarded and 500 µl of buffer AW1 (Qiagen) added, followed by centrifugation at 6000 x g for 1 min. Again the flow-through was discarded and 500 µl of buffer AW2 (Qiagen) added. After centrifugation at 20000 x g for 3 min and discarding the flow-through, the bound DNA was eluted by addition of 200 µl buffer AE (Qiagen), incubation for 1 min and centrifugation at 6000 x g for 1 min at room temperature. The DNA concentration was determined as described in 4.1.5.

4.1.14 Isolation of cellular RNA

The QIAGEN RNeasy Mini Kit was used for isolation of total cellular RNA. Up to $1x10^6$ cells were treated in one purification step. First, cells were trypsinised (see materials and methods 4.3.2) and harvested by centrifugation at 300 x g for 5 min. The supernatant was aspirated completely and cells were disrupted by adding 350 µl of buffer RLT (Qiagen) followed by vortexing. For fragmentation of genomic DNA, the lysate was transferred into a QIAshredder spin column (Qiagen) and centrifuged for 2 min at full speed (Eppendorf table

top centrifuge). 350 µl of 70 % ethanol were added, mixed well by pipetting and transferred to an RNeasy spin column (Qiagen) followed by centrifugation at 8000 x g for 15 sec. After discarding the flow-through, 700 µl of buffer RW1 (Qiagen) were added and again centrifugation was performed at 8000 x g for 15 seconds. Flow-through was discarded. 500 µl of buffer RPE (Qiagen) were added and centrifugation performed at 8000 x g for 2 min. Flow-through was discarded and, to get rid of any residual buffer, centrifugation was performed again at full speed for 1 min. The RNeasy spin column was placed in a new 1.5 ml polypropylene tube, 50 µl of RNase-free ddH_2O (Qiagen) were added directly to the membrane and RNA eluted by centrifugation at 8000 x g for 1 minute. Concentration of RNA was determined as described in 4.1.5.

4.1.15 *In vitro* transcription of RNA

For the stepwise approach of establishing the StreptoTag method from *in vitro* to *in vivo* conditions, first, *in vitro* transcribed RNA was used. RNA was produced using the T7 RiboMAX Express Large Scale RNA production system (Promega). Prior to RNA synthesis, the DNA template had to be linearised (see materials and methods 4.1.2) in order to produce a run-off transcript starting at the T7 promoter of the RNA encoding vector. After linearisation, the DNA was purified from the agarose gel (see materials and methods 4.1.4). Up to 1 µg of DNA was used as a template. Components were mixed gently and incubated at 37°C for 1 hour followed by RNA cleanup (see materials and methods 4.1.14) and determination of RNA concentration (see materials and methods 4.1.5). *In vitro* transcribed RNA was stored in aliquots of 100 µg at -80°C.

T7 Reaction Components :	10 µl	RiboMAX 2 x buffer
	1 µg	linear DNA template
	2 µl	T7 Enzyme Mix
	ddH_2O up to 20 µl	

4.1.16 Denaturing RNA gel electrophoresis

A denaturing RNA gel electrophoresis was performed because most RNAs form complex secondary structures by intramolecular base pairing and therefore do not migrate through the gel strictly according to their size. By applying an electric current, negatively charged nucleic acids migrate through the agarose gel to the cathode, corresponding to the expected size of the fragment to be analysed. Smaller fragments are less retained in movement and therefore migrate faster than larger ones. For preparation of the gel, 2 g of agarose were mixed with 180 ml of DEPC-treated water followed by cooking in a microwave oven at 750 W until the agarose was dissolved completely. The gel was left on the magnetic stirrer for 10 min to cool down below 50°C and then 20 ml of 10x MOPS as well as 7 ml formaldehyde were added. Before pouring the gel, the chamber had to be washed and maintained RNAse free. Therefore, the chamber was incubated 10 min with 5% SDS and then washed with ddH_2O. After incubation with 3% H_2O_2 for further 10 min, the chamber was washed twice with DEPC ddH_2O. Upon this procedure, the previously prepared gel was poured into the chamber and allowed to solidify. As running buffer 1x MOPS in DEPC-treated ddH_2O was used. The RNA samples were mixed with a denaturing 2x RNA loading dye (Fermentas) and heated for 10 min at 70°C followed by centrifugation to collect evaporated sample which were kept on ice until loading.

DEPC-treated water :	5 ml	Diethylpyrocarbonate
	5 l	ddH_2O water
	stirring	
	autoclave	
10x MOPS :	41.8 g	MOPS
	4.1 g	NaAc
	40 ml	0.5 M EDTA pH=8
	fill to 1 l with DEPC ddH_2O => pH=7 (NaOH)	

4.2 Purification of recombinant proteins by affinity chromatography

4.2.1 Expression of recombinant proteins

Large scale expression of recombinant proteins for subsequent automated His-tag affinity purification was carried out in Escherichia coli BL21(DE3). Expression was driven by a T7 promoter, controlled by an Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible lac-operon. First, 50 µl of an already characterised single clone of transformed bacteria were incubated in 2 ml LB containing the appropriate antibiotic at 37°C on a shaker at 200 rpm. After 2 hours, a total of 2 ml were used to inoculate 75 ml of LB medium, also containing antibiotics for selection, which were then incubated at 37°C over night with continuous shaking at 170 rpm in a 0.5 l Erlenmeyer flask. The following day, 5 ml of the over night culture were used to inoculate 200 ml LB medium without antibiotics in a 1 l flask. At OD600 = 0.6, an aliquot of 1 ml was taken out (non-induced control) and IPTG was added to the 200 ml culture to a final concentration of 1 mM. Induction was carried out by incubation at 37°C with 175 rpm on an orbital shaker. After 3 hours, bacteria were harvested by centrifugation at 6000 x g for 15 min at 4°C. The resulting pellet of approximately 1 g in weight was either frozen at -20°C for mid-term storage or directly processed for protein purification.

4.2.2 Preparation of bacterial samples for automated His-Tag affinity purification

The bacterial pellet was resuspended in 5 ml of washing buffer (see materials and methods 4.2.4) and after addition of 50 µl of protease inhibitor cocktail (Sigma-Aldrich P8340), lysozyme was added to a final concentration of 2 mg/ml. The bacterial suspension was vortexed briefly, then shaked at room temperature for 20 min. For further destruction of bacteria, they were sonified for 2 min on ice using the Bandelin UW2070 with a MS73 tip followed by a 10

sec vortex step and shaking on ice for 10 min. This procedure was carried out twice. $MgCl_2$ was then added to a final concentration of 20 mM per gram tissue as well as Benzonase nuclease (DNAse/RNAse, Novagen) to a final concentration of 500 units per gram tissue. The mixture was again incubated under vigorous shaking for 15 min at room temperature. Bacterial debris was spun down by centrifugation at 18000 x g for 20 min at 4°C. To prevent clogging of the column, the protein containing supernatant was filtered through a 0.45 nm filter (Millipore) and finally diluted in 25 ml of washing buffer. To increase the yield of purified protein, the expression approach was carried out in duplicates, which were combined after the filtration step and diluted in 50 ml washing buffer. The total amount was loaded into the ÄKTAprime superloop and the purification carried out as described in 4.2.4. Fractions were stored at 4°C until those containing the protein of interest were determined by Western blot analysis (see materials and methods 4.6).

4.2.3 Technical equipment : ÄKTAprime plus FPLC

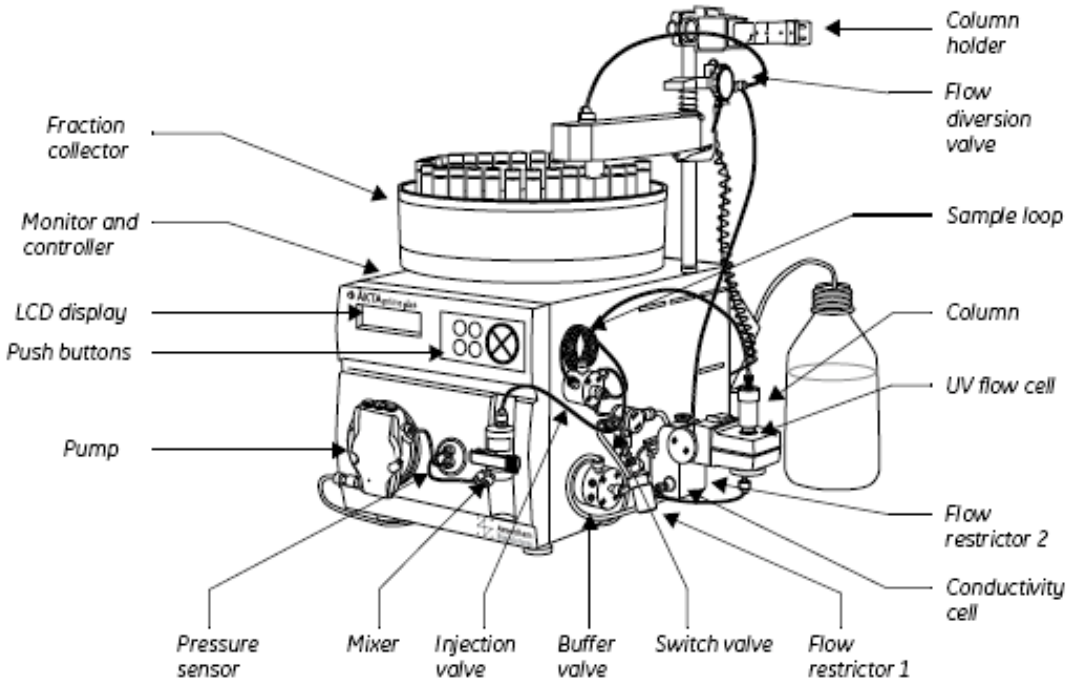


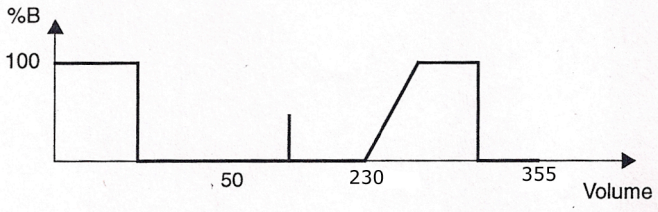
Figure 4.2.3. Schematic view of ÄKTAprime plus FPLC. The unit includes the control system, a pump, a fraction collector, and valves for buffer selection, sample injection, gradient formation and flow diversion. Furthermore, the unit used was equipped with a superloop placed in the column holder to allow application of samples of up to 50 ml (from ÄKTAprime plus Operating Instructions 28-9597-89 AA).

ÄKTAprime plus is a compact fast protein liquid chromatography system for purification of proteins in lab scale. Recombinant protein was obtained by inducing expression in transformed bacteria (for expression plasmids see appendix 8) as described in 4.2.1, followed by isolation of total protein from bacteria as described in 4.2.2.

4.2.4 His-Tag affinity purification

Recombinant proteins carrying a polyhistidine-tag (His-Tag) can be purified by their interaction with metal ions which are contained in the affinity medium (Hochuli 1988; Hengen 1995). For native purification of histidine-tagged proteins, the sample was injected in a volume of 50 ml into the injection valve

and loaded into the superloop. Affinity purification was carried out automatically using a 5 ml HisTrap FF pre-packed column (GE Healthcare) by running a predefined program depicted below. To avoid copurification of bacterial proteins, the recombinant His-tagged protein was purified with stringent conditions using buffers containing imidazole in concentrations of 200 mM (washing buffer) and 1 M (elution buffer), respectively.



Action	Volume	Conc %B	Flow	Fract.	Buffer V	Inject V
Priming B	0	100	40	0	pos 1	WASTE
End priming B	25	100	40	0	pos 1	WASTE
Priming A1	25+0.1	0	40	0	pos 1	WASTE
Equilibration	60	0	5	0	pos 1	LOAD
Autozero	95					
Sample application	95	0	2.5	0	pos 1	INJECT
Wash 3	145	0	5	0	pos 1	LOAD
Elution	230	0	2.5	3	pos 1	LOAD
Wash 4	280	100	5	5	pos 1	LOAD
End wash 4	310	100	5	0	pos 1	LOAD
Priming A2	310	0	40	0	pos 1	WASTE
Re-equilibration	325	0	5	0	pos 1	LOAD
End method	355					

Figure 4.2.4. Affinity chromatography using a His-Tag. Method performed on a ÄKTAprime plus for native purification of histidine-tagged proteins. Volume is given in ml and flow in ml/min (from ÄKTAprime plus User Manual 11-0026-44 AA).

Washing buffer :	20 mM	Sodium phosphate buffer
		pH 7.4
	0.5 M	NaCl
	200 mM	Imidazole

Elution buffer :	20 mM	Sodium phosphate buffer pH 7.4
	0.5 M	NaCl
	1 M	Imidazole

4.2.5 Concentration of fractionated protein samples

Fractions containing high amounts of protein of interest were determined by Western blot analysis (see materials and methods 4.6). Protein from those fractions was concentrated using centrifugal concentration units (Vivaspin) with a molecular weight cut off (MWCO) of 3 kDa. The unit was filled with the respective eluate and centrifugation was performed in a swinging bucket rotor at 5000 x g at 4°C. Finally fractions were concentrated by consecutive centrifugation steps, the imidazole-containing elution buffer was exchanged by a 20 mM sodium phosphate buffer to ensure protein stability. Protein concentration was determined in a Lowry assay (see materials and methods 4.2.6). The concentrate of about 200 µl was aliquoted and stored as described in 4.2.7.

4.2.6 Determination of protein concentrations

Protein concentration was determined using a Lowry protein assay kit (Bio-Rad), (Lowry, Rosebrough et al. 1951). The higher the protein content in a given solution, the stronger the detectable change in color caused by reduction of copper ions through peptide bonds. Therefore 5 µl of each sample were pipetted in duplicates into a 96 well plate (Sarstedt - flat bottom) as well as the standard consisting of bovine serum albumin (BSA) in a range from 0.1 to 1.0 µg/µl. 25 µl of reagent A (Bio-Rad), an alkaline copper tartrate solution, supplemented with 2% of reagent S (Bio-Rad) was added, followed by addition of 200 µl of reagent B (Bio-Rad). The samples were incubated for 20 min at room temperature and absorbance was measured at 630 nm. Protein concentration was calculated comparing OD values of samples to those of the standard containing known BSA concentrations.

4.2.7 Long term storage of proteins

For long term storage, proteins in 20 mM sodium phosphate buffer pH = 7.4 were mixed with glycerol to a final concentration of 25% to preserve functionality after thawing. Aliquots were stored at -80°C.

4.3 Cultivation of eukaryotic cells

4.3.1 Cell lines

HEK293 cells (ATCC CRL-1573) :
The HEK293 cells are a hypotriploid cell line originating from human embryonic kidneys, which was established by transformation with adenovirus type 5 (Louis, Evelegh et al. 1997). A subcultivation of 1:10 to 1:20 and a medium renewal 2-3 times per week is recommended by ATCC.

CrFK cells (ATCC CCL-94) :
This cell line is derived from feline kidneys (Crandell, Fabricant et al. 1973). A subcultivation of 1:3 to 1:8 and a medium renewal 2-3 times per week is recommended by ATCC.

4.3.2 Cultivation

Normal medium (NM) :	Dulbecco's modified eagle medium (DMEM) with high glucose (GlutaMAX) and 10% heat inactivated Fetal Calf Serum (FCS)

Trypsin-EDTA :	8.0 g/l	NaCl
	0.4 g/l	KCl
	0.35 g/l	$NaHCO_3$
	0.726 g/l	$Na_2HPO_4 – 7H_2O$
	1.0 g/l	D-Glucose
	0.2 g/l	EDTA
	0.5 g/l	Trypsin
	0.005 g/l	Phenol red in ddH_2O
Phosphate buffered saline (PBS) :	1.06 mM	KH_2PO_4
	155.17 mM	NaCl
	2.97 mM	$Na_2HPO_4 – 7H_2O$
	pH 7.4	

All working steps with cells were carried out under sterile conditions using a laminar air flow hood (Nuaire), and single-use liquid handling equipment (Sarstedt). The laminar air flow hood as well as all equipment used was cleaned prior to work using 70% ethanol, and the hood was sterilised afterwards by exposure to UV radiation for 20 min.

Media, FCS and trypsin were purchased from Invitrogen. Cells were cultured in polypropylene plastic flasks T75 (75 cm^2) or T175 (175 cm^2); (Greiner or Sarstedt) with a specially treated surface to facilitate cell adherence. Phenol red added to the normal medium served as an indicator for pH change. A yellow shift indicated an acidic environment and a medium change had to be performed. The cells were cultivated at 37°C with an atmosphere containing 5% CO_2.

To passage cells, the old medium was removed first and cells washed with 5 ml of PBS to get rid of excess FCS which lowers activity of trypsin which is used to detach cells by digesting cell-cell and cell-matrix interactions. 3-4 ml of trypsin-EDTA were incubated with the cells at 37°C for 5 min. Protease activity was neutralised by adding an excess of normal medium containing 10% FCS. Cells were split in ratios as recommended by the supplier, the american type culture collection (ATCC). One part of the cells was discarded and the other incubated in fresh medium. This procedure was defined as one passage.

4.3.3 Determination of cell number

To achieve a high transfection efficiency, an exact density of seeded cells is a prerequisite. Therefore, trypsinised cells were resuspended properly in 10 ml of medium. 100 µl were diluted in 900 µl of medium and thereof one drop was loaded in duplicates to a 16 square disposable haemocytometer (FastRead, Megumed Diagnostics). 2 x 3 squares were counted using a microscope (Olympus) and the average value calculated. This number was multiplied with 10^4 to equal the number of cells contained in 1 ml of the solution.

4.3.4 Freezing and thawing

To ensure integrity of cells used, they were discarded after 30 passages and therefore a frozen stock of cells had to be kept. The stock was prepared by harvesting cells grown to confluence in a T175 cell culture flask. After detaching, cells were centrifuged at 300 x g for 5 min, washed with 10 ml PBS and centrifuged again following resuspension in 3 ml of freezing medium. Aliquots of 1 ml were prepared and slowly cooled from room temperature to 0°C on blue ice for 30 min. A freezing container (“Mr.Frosty”, Nalgene) was used to cool cells down to -80°C at a cooling rate of -1°C/min which is required for successful cell cryopreservation.
Cells were thawed by resuspension in warm normal medium, followed by centrifugation at 300 x g for 5 min to get rid of dimethyl sulfoxide (DMSO). The pellet was resuspended in 10 ml normal medium and transferred to a T75 flask.

Freezing medium :	10%	Dimethylsulfoxide (DMSO)
	30%	FCS
	60%	Normal medium (NM)

4.3.5 Transfection

The process known as transfection is used to introduce heterogenic DNA or RNA into cells. To facilitate endocytosis of DNA into cells, transfection reagents such as cationic lipids are used which form complexes with negatively charged DNA. Once in the cytoplasm, uptaken plasmid DNA can access the nucleus during cell division where any gene driven by a mammalian promoter will be transcribed.
Lipofectamine 2000 (Invitrogen) was used as a transfection reagent. All transfections were carried out with cells grown either in 10 cm dishes or in 6-well plates (Sarstedt). Cells were seeded 24 hours prior to transfection and further grown to 90-95% confluence to allow efficient transfection. A maximum of 25 µg of DNA and 60 µl of Lipofectamine 2000 were incubated separately in 1.5 ml of FCS-free DMEM. After 5 min, DNA was mixed with the transfection reagent and incubated for further 20 min to allow formation of DNA-lipofectamine complexes. Subsequently the solution was applied dropwise to the cells and mixed by shaking the plate 5 times cross-wise. After 8 hours, the medium was exchanged to remove excessive Lipofectamine 2000. Transfected cells treated this way were analysed 48 hours later.

4.3.6 Crude preparation of cell lysates

For isolation of proteins and RNA from eukaryotic cells, cytoplasmic fractions and nuclear cell fractions were prepared.
Transfected HEK293 cells grown in a 10 cm dish were trypsinized (see materials and methods 4.3.2) and centrifuged at 300 x g for 5 min. Medium was removed, cells washed with 5 ml of PBS and centrifuged again. The cell pellet was resuspended in 200 µl of lysis buffer containing octylphenoxypolyethoxyethanol (IGEPAL - Sigma) as a detergent. 8 µl of RNAsin plus RNAse inhibitor (40 U/µl - Promega) and 5 µl of protease inhibitor cocktail (Sigma-Aldrich P8340) were added and the sample vortexed immediately until cells were lysed completely and the solution turned opaque. The lysate was centrifuged at 3000 x g for 5 min at 4°C and the supernatant

containing the cytoplasmic fraction was stored at 4°C. The pellet containing the nuclei was substituted with 4 µl RNAsin Plus RNAse Inhibitor and 5 µl of protease inhibitor cocktail (Sigma-Aldrich P8340) and 100 µl of protein isolation buffer were added to resuspend the nuclear fraction. For proper lysis, the solution was kept on ice and vortexed every 5 min for 10 seconds over 1 hour. Cellular debris was spun down at 12000 x g for 15 min at 4°C and the supernatant was transferred to a new tube. Fractions were unified, incubated for 15 min and loaded onto the column.

IGEPAL lysis buffer :	50 mM	Tris-HCl pH 7.5
	140 mM	NaCl
	1.5 mM	$MgCl_2$
	0.5%	IGEPAL
	5 mM	Dithiothreitol (DTT) (freshly added)
Protein isolation buffer :	10 mM	Tris-HCl pH 7.8
	0.4 M	NaCl
	1 mM	DTT (freshly added)
	0.2 mM	Phenylmethanesulfonyl fluoride (PMSF) (freshly added)

4.4 Fluorescence activated cell sorting (FACS)

To ensure a maximum expression level of the protein and / or RNA to be purified by affinity chromatography, transfection efficiency needed to be sufficiently high. To determine transfection efficiencies, cells were co-transfected with pCMVGFP, an expression vector encoding green fluorescent protein, in addition to the expression plasmids encoding the protein and RNA of interest. The amount of fluorescent cells, which represents the number of successfully transfected cells, was determined using a FACSCalibur (Becton, Dickinson) flow cytometer.

Measurement was carried out by exciting fluorescence using a monochromic blue argon laser (488 nm wave length). The number of fluorescent cells detected divided by the over all amount of cells measured was referred to as efficiency of transfection.

4.5 StreptoTag affinity purification

After nuclear/cytoplasmic fractionation of cells the lysate was subjected to affinity chromatographic purification using a streptomycin-coupled sepharose.

4.5.1 Coupling of sepharose with streptomycin

For the coupling reaction, 5 g of epoxy-activated sepharose 6B (GE Healthcare) were dissolved in 1 l of cell culture grade H_2O and incubated for 1 hour at room temperature with gentle movement on a magnetic stirrer. Thereafter, the dispersion was filtered through a 40 – 100 µm sintered glass filter (Schott) and the sepharose resuspended in 50 ml of 98% ethanol followed by four consecutive washing steps with 50 ml of coupling buffer. The washed column material was incubated for 16 hours in 25 ml of coupling buffer containing 1 mM of dihydrostreptomycin with gentle movement on an orbital shaker at 37°C. Dihydrostreptomycin (Sigma) was used as the coupling reaction occurs in 10 mM NaOH and streptomycin would undergo irreversible hydrolysis under these pH conditions (Bachler, Schroeder et al. 1999). Again, coupled sepharose was filtered through a 40 – 100 µm sintered glass filter and washed four times with 50 ml of column buffer. For regeneration of the coupled sepharose, alternating washing steps were carried out using a low pH-buffer (pH 4.0) and a high pH-buffer (pH 8.3) three times each. Finally, the dihydrostreptomycin-coupled sepharose was washed four times with 50 ml cell culture grade H_2O and stored in 50 ml of ddH_2O at 4°C. To prevent bacterial growth, 250 µl of 10% sodium azide were added. Column material was used within one month after coupling.

5 x Column buffer :	25 mM	$MgCl_2$
	250 mM	Tris-HCl (pH 7.6)
	1.25 M	NaCl
Coupling buffer :	10 mM	NaOH
	pH 12.0	
Low pH buffer :	0.1 M	Sodium acetate
	0.5 M	NaCl
	pH 4.0	
High pH buffer :	0.1 M	Sodium hydrogen carbonate
	0.5 M	NaCl
	pH 8.3	

4.5.2 Affinity purification of RNA binding proteins

Prior to filling the coupled sepharose into the Poly-Prep columns (9 cm high, 2 ml bed volume, 10 ml reservoir; Bio-Rad), the column was flushed with 5 ml of column buffer. 2 ml of the coupled sepharose were pipetted into the column and flushed four times with 2 ml of column buffer. To block unspecific RNA binding sites, 10 µg of tRNA diluted in 1 ml of column buffer were applied to the column. When *in vitro* transcribed RNA was used for affinity purification, RNA was refolded after thawing by incubation at 65°C for 8 min, 37°C for 8 min and finally at room temperature for 8 min. 100 µg of tRNA were added to the protein solution and unified with the refolded target RNA. In the next step, RNA and protein mixtures were combined followed by incubation for 20 min at room temperature to allow RNA - protein interaction to occur. The solution was diluted in column buffer to a final volume of 4 ml and loaded onto the column in 4 aliquots to 1 ml each within 8 min. The flow-through of 4 ml was kept and applied in a single step a second time to enhance the amount of RNA and protein retained. Five washing steps with 2.5 ml of column buffer were performed prior to elution with 3 ml of 1 mM streptomycin in column buffer.

Fractions were precipitated with acetone (see materials and methods 4.5.3). Used columns were washed with 8 ml of column buffer and stored in 3 ml of column buffer at 4°C.

4.5.3 Concentration of fractionated proteins

Protein contained in the different fractions eluted from streptomycin-coupled sepharose was concentrated by adding 4 volumes of ice cold (-20°C) acetone. Glass tubes (15 ml, Corex) were sealed with a foil (Parafilm), shaked vigorously and quickly put on -80°C for 30 min. After incubation at -20°C over night, the protein precipitated was instantly centrifuged at 4°C for 30 min at 25000 x g (Avanti J25 - Beckman - Coulter) using a swinging bucket rotor. Supernatant was discarded carefully and the pellet was dried in a vacuum dryer for 5 min and resuspended in 40 µl of 1 x protein sample buffer. The sample was stored at -20°C for further analysis.

4.6 Western blot analysis

4.6.1 SDS-polyacrylamide gel electrophoresis

A sodium dodecyl sulfate (SDS)-polyacrylamide gel is used for separation of proteins. It usually consists of two functional parts: the resolving gel, in which proteins are seperated, is covered by a stacking gel in which samples are loaded and concentrated. For the present work, 12% acrylamide was used for the resolving gel and 5% for the preparation of the stacking gel.
Preparation of SDS-polyacrylamide gels was carried out in a Hoefer SE 260 Mighty Small II (GE Healthcare) dual gel casting unit. After pouring the resolving gel, it was covered by 1 ml of isopropanol to prevent it from drying. Polymerisation was allowed for about 20 min and then the gel was washed with ddH_2O to remove the isopropanol and finally traces of H_2O were removed using a Whatman paper (3MM Whatman). Subsequently, the stacking gel was

poured and allowed to polymerise for 20 min. Finally, gels were placed in a Hoefer Mighty Small II SE 250/260 mini vertical electrophoresis unit (GE Healthcare) and submergered in 1 x Laemmli buffer.
The samples were mixed 1:1 with a 2 x protein sample buffer and heated at 95°C for 5 min to irreversibly denature secondary structures of proteins. Up to 40 µl were loaded into each slot, except of the marker (PageRuler prestained protein ladder - Fermentas), of which 3 µl were used. Electrophoresis of a 12% gel was performed at 90 V for exactly 1 hour and 50 min.

Stacking gel (5%) :	3.75 ml	ddH_2O
	625 µl	Tris-base pH 6.8
	625 µl	40 % acrylamide 29:1 (Bio-Rad)
	25 µl	20% SDS
	30 µl	10% Ammoniumpersulfate (APS)
	10 µl	Tetramethylethylenediamine (TEMED, Bio-Rad)
Resolving gel (12%) :	3.7 ml	ddH_2O
	4.8 ml	Tris-base pH 8.8
	3.6 ml	40 % acrylamide 29:1 (Bio-Rad)
	64 µl	20% SDS
	66 µl	10% APS
	10 µl	TEMED (Bio-Rad)
10 x Laemmli buffer :	0.25 M	Tris-base
	1.92 M	Glycin
	1%	SDS (w/v)
Protein sample buffer :	0.12 M	Tris-HCl pH 6.8
	4%	SDS (w/v)
	20.23%	Glycerol (w/v)
	0.016%	Bromphenol blue (w/v)
	1.47 M	β-Mercaptoethanol

4.6.2 Blotting and immunostaining

Blotting of the gel was performed in a Hoefer TE70 semi dry transfer unit (GE Healthcare). Therefore, 6 pieces of Whatman paper in the same size as the gel (62 mm x 83 mm) and one 0.45 µm Hybond P Polyvinylidne fluoride (PVDF) transfer membrane (GE Healthcare) were prepared. The stacking gel was removed and the resolving gel washed in transfer buffer for 5 min. The membrane was first soaked in pure methanol for 15 seconds, following a 5 minute washing step in ddH_2O and finally 5 min of washing in transfer buffer. The blotting sandwich was built by soaking 3 Whatman paper in transfer buffer and placing them on to the transfer unit, followed by the transfer membrane, the gel and another three soaked Whatman papers. To prevent formation of air bubbles, half of a disposable 10 ml polypropylene pipette (Sarstedt) was used to roll over each layer of the sandwich. Blotting was carried out at 20 V (upper limit) and 1.1 mA/cm^2 of membrane for 1 hour and 10 min.

After blotting, the membrane was blocked over night in 5 % milk powder in TTBS at 4°C on a horizontal shaker. The gel was stained with AgNO3 as described in materials and methods, 4.6.4. The next day, the membrane was directly incubated with the primary antibody which was diluted in 5 ml TTBS containing 2.5% milk powder. Placed in a 50 ml polypropylene tube (Falcon), the membrane was incubated in a rotator (Biometra Compact Line OV4) at room temperature. After 1 hour of incubation, the membrane was washed with TTBS 5 times, followed by incubation with the horseradish peroxidase (HRP)-conjugated secondary antibody. Also the secondary antibody was diluted in 5 ml TTBS containing 2.5% milk powder. After one hour of incubation at room temperature, the membrane was washed 3 times with TTBS and 2 times with TBS (Tris-buffered saline) to get rid of excess Tween-20 that could interfere with the detection of chemiluminescence.

For detection of HRP-conjugated secondary antibodies bound to primary antibodies that are interacting with proteins blotted onto the membrane, the ECL-plus Western blot detection system (GE Healthcare) was used. For each membrane, 2 ml of solution A (ECL Plus substrate solution containing Tris-HCl buffer) were mixed with 50 µl of solution B (acridian stock solution in dioxane and ethanol) and warmed up to room temperature. The membrane was

carefully rinsed with the solution for 5 min in a plastic tray and transferred to a transparent plastic foil which was placed in a developing film cassette. Finally the membrane was exposed to an X-ray film (Hyperfilm ECL - GE Healthcare) for an appropriate period of time. The film was developed using an AGFA Curix 60 automatic developer.

Transfer buffer :	192 mM	Glycine
	25 mM	Tris-base
	20%	Methanol (v/v)
	pH 8.3	
Blocking solution :	5%	Non-fat dry milk
	in 1 x TTBS	
10 x TTBS :	0.1 M	NaCl (w/v)
	20 mM	Tris-base pH 8.8
	0.01%	Tween-20 (Bio-Rad)
10 x TBS :	0.1 M	NaCl (w/v)
	20 mM	Tris-base pH 8.8

4.6.3 Silver staining of proteins

After semidry blotting, the gel was stained with AgNO3. This is a fast and very sensitive method for detection of proteins. The gel was fixed in 50% methanol and 5% acetic acid for 20 min on a shaker followed by incubation in 50% methanol for 10 min. The gel was then washed with ddH_2O over night. On the next day the gel was sensitised in 0.02% $Na_2S_2O_3$ for 1 minute followed by 2x washing in ddH_2O for 1 minute each. Afterwards, the gel was incubated in cold 0.1% $AgNO_3$ for 20 min. To remove excess $AgNO_3$ two washing steps in ddH_2O for 1 minute each were performed. Finally, the gel was developed in 0.04% formalin and 2% Na_2CO_3 for 2-5 min. Staining of the gel was terminated

by incubation in 5% acetic acid for 10 min. For mid-term use, the gel was stored at 4°C in 1% acetic acid.

4.7 Polymerase chain reactions

4.7.1 Primer design

Oligonucleotide primers for use in polymerase chain reaction (PCR) amplification were designed virtually using the software Clone Manager 5 as described in materials and methods, 4.1.1. Specifications for design of primers were given by the guanosine to cytosine content which should range between 50% and 60%. Primer sequences were submitted online and ordered from Microsynth (Switzerland).

4.7.2 PCR amplification

For selectively amplifying a specific DNA sequence, PCR was performed using specifically designed primers. For performing analytical PCR, Taq DNA polymerase derived from Thermus aquaticus, without proof-reading activity was used.

PCR master mix for Taq DNA polymerase :

10 mM	each dNTP
100 pmol/µl	forward and reverse primers
10 µl	5 x GoTaq buffer (Promega)
2 U	Taq DNA polymerase
5 – 10 ng	DNA template
ddH_2O up to 50 µl	

For PCR amplification either the Gene Amp PCR System 9700 (Applied Biosystems) or peqSTAR 96 (Peqlab) were used. For analytical PCR the

following standard amplification was carried out (step 1, denaturation; step 2, denaturation-primer annealing-elongation; step 3, elongation; step 4, storage) :

Profile	step 1	step 2 (x 30)			step 3	step 4
Temp	95°C	95°C	55°C	72°C	72°C	4°C
Time	2 min	30 sec	30 sec	1 min	7 min	hold

Subsequently up to 20 µl were withdrawn for analysis by agarose gel electrophoresis as described in materials and methods, 4.1.3.

4.7.3 cDNA synthesis

To amplify RNA in PCR, first it had to be reversely transcribed into DNA. Therefore, total cellular RNA was isolated from eukaryotic cells as described in materials and methods, 4.1.14. To exclude amplification of any residual DNA co-purified during RNA isolation, a DNAse digest was performed prior to cDNA synthesis as described in materials and methods, 4.7.4. The DNA-free solution was then used to reversely transcribe RNA into complementary DNA (cDNA), which subsequently could be amplified by PCR for further analysis. For reverse transcription (RT) the high-capacity cDNA reverse transcription kit (Applied Biosystems) was used.

cDNA master mix :	2 µl	10 x RT buffer
	0.8 µl	25 x dNTP mix (100 mM)
	2 µl	10 x RT random Primers
	1 µl	reverse transcriptase from Moloney murine leukemia virus (MoMuLV)
	4.2 µl	nuclease-free H_2O
	10 µl	RNA template up to 2 µg

To perform reverse transcription of RNA, the following programme was used for amplification in a thermal cycler (step 1, primer annealing; step 2, reverse transcription; step 3, RT inactivation; step 4, storage):

Profile	step 1	step 2	step 3	step 4
Temp	25°C	37°C	85°C	4
Time	10 min	120 min	5 sec	hold

After synthesis of cDNA an aliquot of 1 µl was taken and subjected to PCR (see materials and methods 4.7.2). As a control, a PCR was performed using non reversely transcribed RNA as a template. The amplified product was analysed by agarose gel electrophoresis from both fractions.

4.7.4 DNAse digest

Prior to performing cDNA synthesis with the isolated RNA, a DNAse digest had to be performed, to remove traces of plasmid DNA remaining from previous transfections. In subsequent PCR amplifications presence of plasmid DNA would lead to a false positive signal. As a control for successful digestion, a PCR was performed before and after the digest. In order to exclude any DNA contamination, a double digest was performed using the TURBO DNA-free kit (Applied Biosystems). Up to 0.2 µg DNA/µl were treated in a rigorous DNAse digest. Digests were typically carried out in a reaction volume of 25 µl. 2.4 µl of 10 x TURBO DNAse buffer and 1 µl of TURBO DNAse were added to the RNA and incubated at 37°C. After 30 min, again 1 µl of TURBO DNAse was added and the reaction incubated for further 30 min at 37°C. To stop the digest, 2.6 µl of DNAse inactivation reagent included in the kit, were added and mixed constantly during incubation for 5 min at room temperature. The inactivation reagent was separated by centrifugation at 10000 x g for 1.5 min. The supernatant was carefully transferred to a new tube without touching the pelleted inactivation reagent which interferes with Taq DNA polymerase in subsequent PCR steps.

4.7.5 Semi-quantitative RT-PCR

The semi-quantitative RT-PCR is a method for determining the *in vivo* expression level of recombinant SRS-RNA transcribed from the respective transfected expression vector. By comparing the signal strength generated by amplification of the cDNA in RT-PCR to a standard of reverse transcribed RNA of known amounts (*in vitro* transcribed SRS-RNA) the transcription level can be calculated. To this aim, RNA isolated out of transfected cells was reversely transcribed (see materials and methods 4.7.3) after rigorous DNAse treatment (see materials and methods 4.7.4) and subjected to semi-quantitative PCR. A routine 30 cycle PCR amplification as described in materials and methods 4.7.3, was performed and tubes were removed from the thermocycler every three cycles and analysed on a 1% agarose gel. The signals obtained were compared to those of the standard allowing calculation of the respective cellular RNA expression level.

4.8 Mass spectrometry

In the present work RNA-binding proteins obtained from purification with the *in vivo* StreptoTag method were further analysed by mass spectrometry. Therefore, proteins were subjected to SDS-PAGE, the gel stained with AgNO3 (see materials and methods 4.6.4) and the band of interest excised for submission to mass spectrometry. In this work, mass spectrometrical analysis was carried out by VetOMICS, a spin off company of the university of veterinary medicine Vienna. In MALDI (Matrix Assisted Laser Desorption/Ionisation) protein ions are generated by a laser beam. In an electrical field these ions are accelerated and sent through a flight tube with the lightest being the fastest and arriving at the detector first. The resulting time of flight (TOF) of the ionized peptide is proportional to its mass/charge ratio. For identification of the peptide corresponding protein, the spectrum can be blasted against a database of a given organism.

5. Results

5.1 Affinity purification of recombinant proteins

5.1.1 Purification of recombinant His-tagged HIV-1 Rev protein

To establish the StreptoTag method for *in vivo* applications, a stepwise approach was chosen. In the first step recombinant HIV-1 Rev protein was purified by binding to *in vitro* transcribed SRS-RNA. Second, HIV-1 Rev was cellularly expressed but still isolated by using *in vitro* transcribed SRS-RNA. Finally, protein as well as RNA were expressed in co-transfected cells and directly applied to the column for purification.

In the first step, purification conditions should be standardised using *in vitro* transcribed RNA (see materials and methods 4.1.15) in combination with functional recombinantly produced RNA-binding protein. As a model system, the well known RNA-protein interaction of HIV-1 Rev and its responsive element RRE was applied. Recombinant Rev protein was purified from E.coli transformed with the vector pETsRevCHis (see appendix 8.2) encoding the HIV-1 Rev protein C-terminally tagged with histidine. His-tagged protein was expressed and affinity purified by Ni-chelate chromatography as described in materials and methods, 4.2.4. Aliquots of FPLC-purified His-Rev protein were separated by SDS-PAGE and recombinant protein detected by Western blotting using a sheep α-Rev antibody diluted 1:4500 (Fitzgerald) as a primary, and a mouse anti-sheep antibody conjugated to horseradish peroxidase (HRP) as a secondary antibody (diluted 1:1300; DAKO); (Figure 5.1.1).

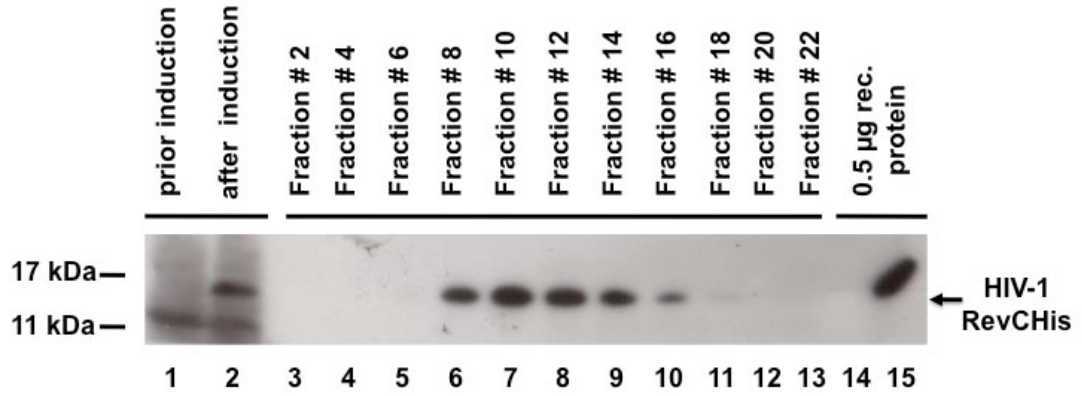


Figure 5.1.1 Western blot of FPLC-purified recombinant HIV-1 RevCHis. Lane 1 : total protein extracts of E.coli harbouring pETsRevCHis prior to induction. **Lane 2 :** total protein extracts of E.coli harbouring pETsRevCHis 3 hours after induction with 1 mM IPTG. **Lane 3 - Lane 13 :** Aliquots of proteins of FPLC fractions 1 - 24. **Lane 14 :** empty. **Lane 15 :** 0.5 µg recombinant RevCHis from a previous purification (positive control).

As shown in Figure 5.1.1, induction of expression of recombinant RevCHis protein was successfully achieved by addition of 1 mM IPTG (compare lane 1 and lane 2). Analysis of the different fractions of the respective FPLC-purification revealed recombinant protein in fractions 8 - 16 (Figure 5.1.1, lane 6 - 10). Fractions 8 - 16 containing RevCHis were concentrated by size exclusion centrifugation units (Vivaspin) as described in materials and methods, 4.5.3. Purified RevCHis protein in size (14 kDa) was identical to a previously purified aliquot (0.5 µg) used as a positive control (Figure 5.1.1, lane 15).

5.1.2 Purification of recombinant His-tagged MMTV Rem(p14) protein

As a second model system for specific RNA-protein interactions, the MMTV Rem/RmRE system was investigated. Therefore, the RmRE binding part of MMTV Rem, the Rem(p14) protein was recombinantly expressed by E.coli containing the pETRemSPCHis vector (see appendix 8.2). His-tagged Rem(p14) was purified applying the same protocol as for affinity purification of HIV-1 RevCHis (see results 5.1.1). Aliquots of fractions eluted from FPLC were analysed by SDS-PAGE and Rem(p14) detected by Western blotting using a monoclonal mouse α-Rem antibody (M66; Hochman, Park et al. 1990)

as a primary and a rabbit α-mouse antibody conjugated to HRP (diluted 1:10000) as a secondary antibody (Figure 5.1.2). The smaller band in lane 14 most probably is degraded Rem(p14) protein due to long term storage.

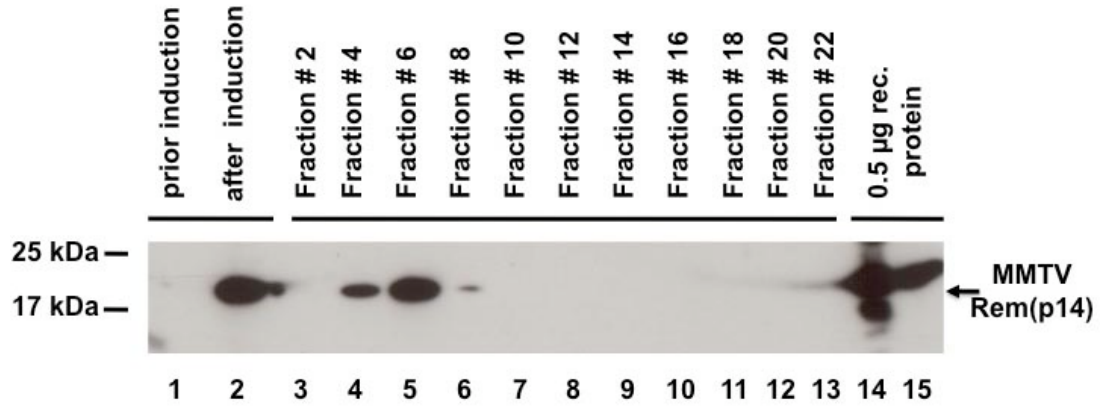


Figure 5.1.2 Western blot of FPLC-purified recombinant MMTV Rem(p14). Lane 1 : total protein extracts of E.coli harbouring pETRemSPCHis prior to induction. **Lane 2 :** total protein extracts of E.coli harbouring pETRemSPCHis 3 hours after induction with 1 mM IPTG. **Lane 3 - Lane 13 :** Aliquots of proteins of FPLC fractions 1 - 24. **Lane 14 :** 0.5 µg recombinant Rem(p14)CHis from a previous purification (positive control). **Lane 15 :** 0.5 µg recombinant Rem(p14)CHis after concentration of FPLC fractions 3 - 9.

As shown in Figure 5.1.2, induction of expression of recombinant Rem(p14)CHis protein was successfully achieved by addition of 1 mM IPTG (compare lane 1 and lane 2). Analysis of the different fractions of the respective FPLC-purification revealed recombinant protein in fractions 4 - 8 (Figure 5.1.2, lane 4 - 6). Fractions 4 - 8 containing Rem(p14)CHis were concentrated by size exclusion centrifugation units (Vivaspin) as described in materials and methods, 4.5.3. Purified Rem(p14)CHis protein in size (19 kDa) was identical to a previously purified aliquot (0.5 µg) used as a positive control (Figure 5.1.2, lane 14).

5.2 *In vitro* transcription of Strepto-tagged RNA

5.2.1 Synthesis of double Strepto-tagged HIV-1 RRE-RNA

To retain recombinantly purified RevCHis protein on streptomycin-coupled sepharose columns, presence of its target RRE-RNA is essential. To facilitate binding of RRE-RNA to streptomycin, it was modified to contain a StreptoTag aptamer at the 5' end as well as at the 3' end (see introduction 3.2.2). The SRS-RNA was transcribed *in vitro* from the pTZSRS vector (see appendix 8.2) linearised with EcoR1 starting from the T7 promoter resulting in a 350 nucleotide long run-off transcript (see materials and methods 4.1.15). The transcribed RNA was analysed by denaturing agarose gel electrophoresis (see materials and methods 4.1.16) to verify molecule length and integrity. As depicted in Figure 5.2.2 lane 1, the *in vitro* transcription was successfully achieved. Full length SRS-RNA was obtained in sufficient quantities in the milligram range, and was used for subsequent affinity purification experiments.

5.2.2 Synthesis of double Strepto-tagged MMTV RmRE-RNA

For affinity column purification of recombinantly produced MMTV Rem(p14), successful *in vitro* transcription of its double Strepto-tagged Rem responsive element, the SRmS RNA, was a crucial point. Therefore, the pTZSRmS vector (see appendix 8.2) was linearised with EcoRI and subjected to *in vitro* transcription using the T7 polymerase (see materials and methods 4.1.15). The resulting product was analysed by denaturing agarose gel electrophoresis (see materials and methods 4.1.16). As shown in Figure 5.2.2 lane 2, however, in contrast to SRS-RNA, only 10% of the synthesised SRmS-RNA appears to be full-length. This amount of 580 nt long full-length transcript was calculated to be insufficient to carry out column purification experiments. Although *in vitro* transcription was modified by increasing incubation time and decreasing incubation temperature, still full-length RNA

could not be produced to sufficient amounts. Similarly, trying different T7-transcription systems such as the T7 RiboMAX Express Large Scale RNA production system (Promega), the TranscriptAid T7 High Yield Transcription Kit (Fermentas) as well as the *In vitro* transcription Kit (Roche), was not successful in producing full-length transcripts.

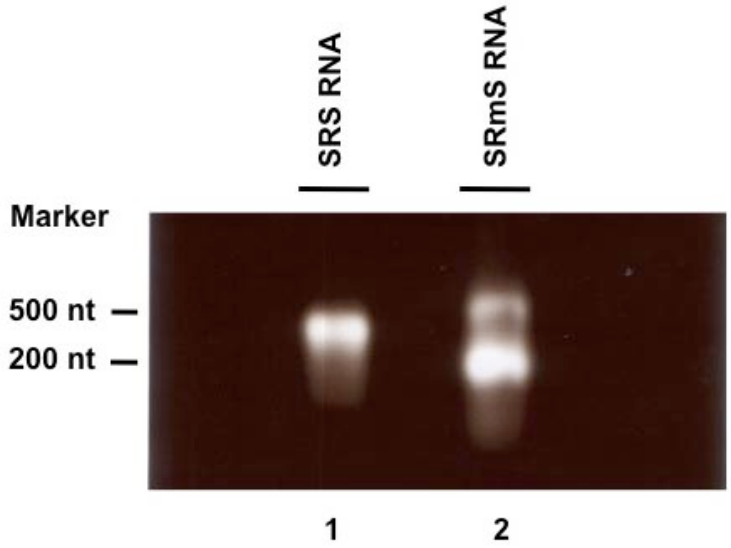


Figure 5.2.2 Denaturing gel electrophoresis of *in vitro* transcribed SRS-RNA and SRmS-RNA. Lane 1 : 0.3 µg of double Strepto-tagged HIV-1 RRE-RNA (SRS) (expected size 350 nt). **Lane 2 :** 0.3 µg of double Strepto-tagged MMTV RmRE RNA (SRmS) (expected size 580 nt).

5.3 *In vivo* transcription of Strepto-tagged viral target RNA

5.3.1 *In vivo* transcription of SRS-RNA

In the stepwise approach of establishing the StreptoTag method for *in vivo* applications, finally cells should be co-transfected with the SRS as well as the Rev encoding vector. For a successful isolation of Rev, full-length transcription of SRS-RNA in transfected cells was crucial and had to be demonstrated. Therefore, total cellular RNA extracted from $1.3x10^6$ HEK293 cells (one well of a 6-well plate), that had been transfected with 5 µg of the SRS encoding vector pCMVSRS (see appendix 8.2), was isolated (see

materials and methods 4.1.14), DNAse-treated (see materials and methods 4.7.4) and reversebly transcribed (see materials and methods 4.7.3). PCR was performed (see materials and methods 4.7.2) using primers binding at the very ends of the 350 base pair long cDNA. Generation of the respective DNA fragment would indicate synthesis of the full-length SRS transcript. As a forward primer, the oligonucleotide 5' - TAT ATC TAG ACA GTG GGA ATA GGA GCT TTG - 3', and as a reverse primer, the oligonucleotide 5' - AGG AGC TGT TGT CCT TTA GGT ATC - 3' was used (primer pair A). The analysis was carried out in duplicates. The obtained PCR products were analysed by agarose gel electrophoresis. The results are shown in Figure 5.3.2. Both PCR reactions (Figure 5.3.2, lane 4 and lane 5) gave rise to a product of 350 nt, corresponding to the full-length transcript of SRS-RNA.

5.3.2 *In vivo* transcription of SRmS-RNA

Similar to verification of *in vivo* full-length transcription of SRS-RNA (see results 5.3.1), analysis of transcription of double Strepto-tagged RmRE-RNA (SRmS) was performed. Therefore, cytoplasmic RNA was isolated from $1.3x10^6$ HEK293 cells transfected with 5 µg of pCMVSRmS (see appendix 8.2). Since *in vitro* transcription of SRmS-RNA had mostly resulted in incomplete transcripts, it was of special interest if SRmS-RNA *in vivo* would be transcribed as a full-length transcript that will allow binding of Rem. Primers for PCR were selected to bind to the very ends of the 580 base pair long cDNA, thus only allowing amplification of reversely transcribed RNA if a full-length transcript has been produced. As a forward primer, the oligonucleotide 5' - CGG ATC CGG AGT GAA GGC TTT AAA TC – 3', and as a reverse primer the oligonucleotide 5' – CAA ATG CGA TCC CTG TGG TCC TTG C – 3' was used (primer pair B). Again, the analysis was carried out in duplicates. As seen in Figure 5.3.2, lane 2 and lane 3, reaction products were analysed by agarose gel electrophoresis. In contrast to *in vitro* transcribed SRmS-RNA, SRmS transcribed *in vivo* is a full-length molecule resulting in the expected fragment of 580 nucleotides in length.

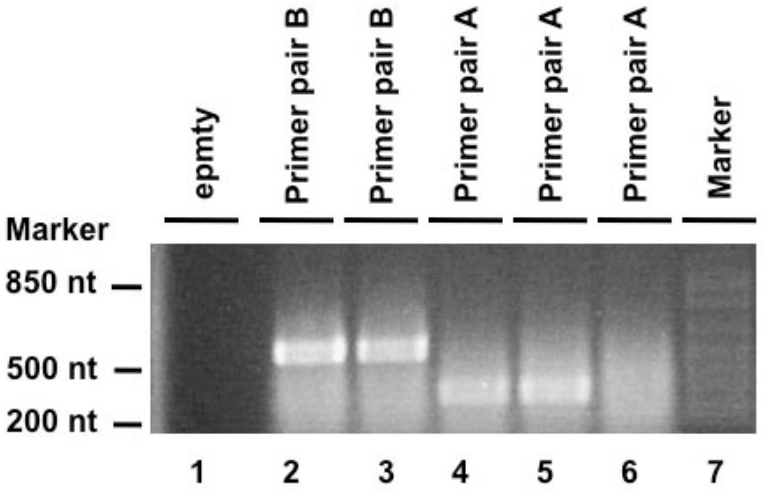


Figure 5.3.2 Agarose gel electrophoresis of PCR fragments. Lane 1 : empty. **Lane 2 and Lane 3 :** PCR products obtained with reversely transcribed cellular SRmS-RNA. **Lane 4 and Lane 5 :** PCR products obtained with reversely transcribed cellular SRS-RNA. **Lane 6 :** no template control reaction. **Lane 7 :** 1 kb plus DNA Marker (Invitrogen).

5.4 Determination of expression levels of cellular SRS- and SRmS-RNA

In order to facilitate an efficient streptomycin-mediated affinity purification of viral RNA/protein complexes, both components had to be produced in sufficient amounts, *in vitro* as well as *in vivo*. Therefore, cellular SRS- as well as SRmS-RNA expression levels were investigated, applying a semi-quantitative RT-PCR approach (see materials and methods 4.7.5). 100 µg of *in vitro* transcribed SRS-RNA were determined to be the required amount to allow StreptoTag-mediated purification of recombinant RevCHis (Müllner Matthias, Diploma Thesis 2007). Therefore, cellular expression of SRS-RNA as well as SRmS-RNA in HEK293 cells transfected with pCMVSRS or pCMVSRmS (see appendix 8.2) should produce similar yields to facilitate successful isolation of cellular Rev. To ensure high expression levels of both, protein and target RNA, the transfection efficiency was of crucial importance.
Therefore, transfection efficiencies were monitored by transfection of a reporter plasmid encoding green fluorescent protein, followed by quantitative evaluation using FACS (Figure 5.4.1, A-D).

Cells transfected with 1 µg of the reporter plasmid pCMVGFP (see appendix 8.2) resulted in a transfection efficiency of 54.61% and cells transfected with 10 µg of the reporter plasmid resulted in a transfection efficiency of 93.90%. According to these results, in all further experiments the reporter plasmid was co-transfected accounting for 10% of the total DNA amount, and only cells exhibiting a minimum transfection efficiency of 55% were further processed.

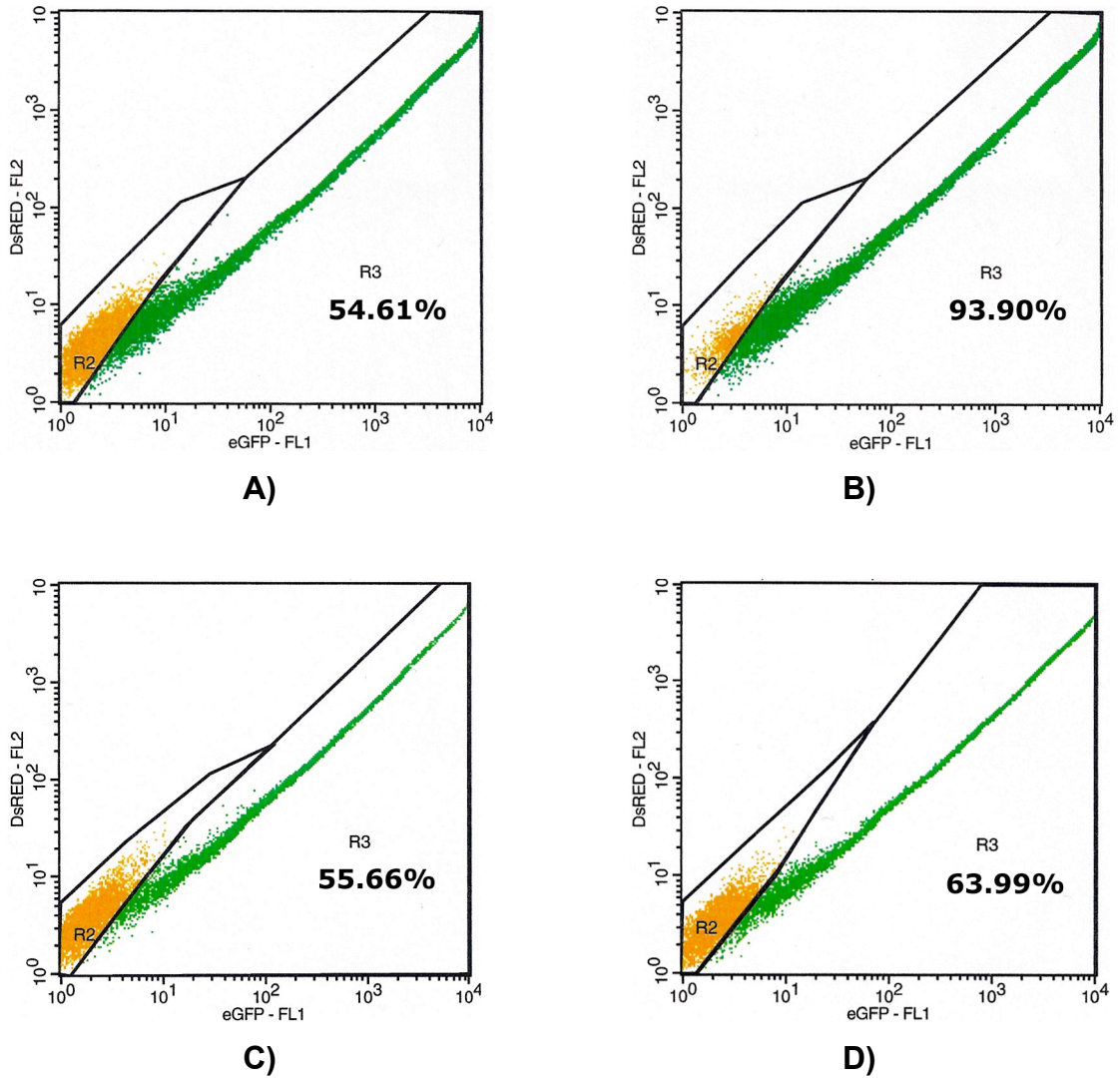


Figure 5.4.1 A) Monitoring of transfection efficiencies : Cells transfected with 1 µg of the reporter plasmid pCMVEGFP show a transfection efficiency of 54.61%. **B**) Cells transfected with 10 µg of the reporter plasmid pCMVEGFP show a transfection efficiency of 93.90%. **C)** Cells co- transfected with pEGFPRev and pCMVSRS show a transfection efficiency of 55.66%. **D)** Cells co-transfected with pEGFPRem and pCMVSRmS show a transfection efficiency of 63.99%.

Transfection efficiencies determined for plasmids encoding HIV-1 Rev, alone or as a fusion protein with EGFP, were shown to be in average about 10% lower compared to constructs coding for MMTV Rem, most probably due to cytotoxic effects. After successful transfection, cellular RNA was prepared and the amount of *in vivo* transcribed SRS- or SRmS-RNA was evaluated by applying semiquantitative RT-PCR (see materials and methods 4.7.5).

As a standard for quantification of obtained RT-PCR products, *in vitro* transcribed SRS-RNA in amounts of 0.1 µg up to 0.7 µg was used (Figure 5.4.2, panel G - panel J). By comparing the band intensities obtained after RT-PCR of *in vivo* transcribed SRS- and SRmS-RNA with PCR products synthesised from the *in vitro* transcribed SRS-RNA standard, the respective amount of RNA was calculated. Quantification of cellular SRS and SRmS expression levels was carried out in duplicates, to improve calculation accuracy. The bands of *in vivo* transcribed SRS (Figure 5.4.2, panel E - lane 8 and panel F - lane 8) and SRmS-RNA (Figure 5.4.2, panel C - lane 8 and panel D - lane 8) arising in cycle #30 correspond to the signal obtained with 0.1 µg of standard RNA (Figure 5.4.2, panel J - lane 8). Corrected by the dilution factors in PCR (x 19), reverse transcription (x 10) and DNAse digest (x 5) reactions, this amount equals a cellular expression level of 95 µg per $1.3x10^6$ cells. Thus, transcription of SRS- and SRmS-RNA from transfected expression vectors should provide a sufficient amount of RNA to facilitate isolation of the respective binding proteins in affinity chromatographic purifications.

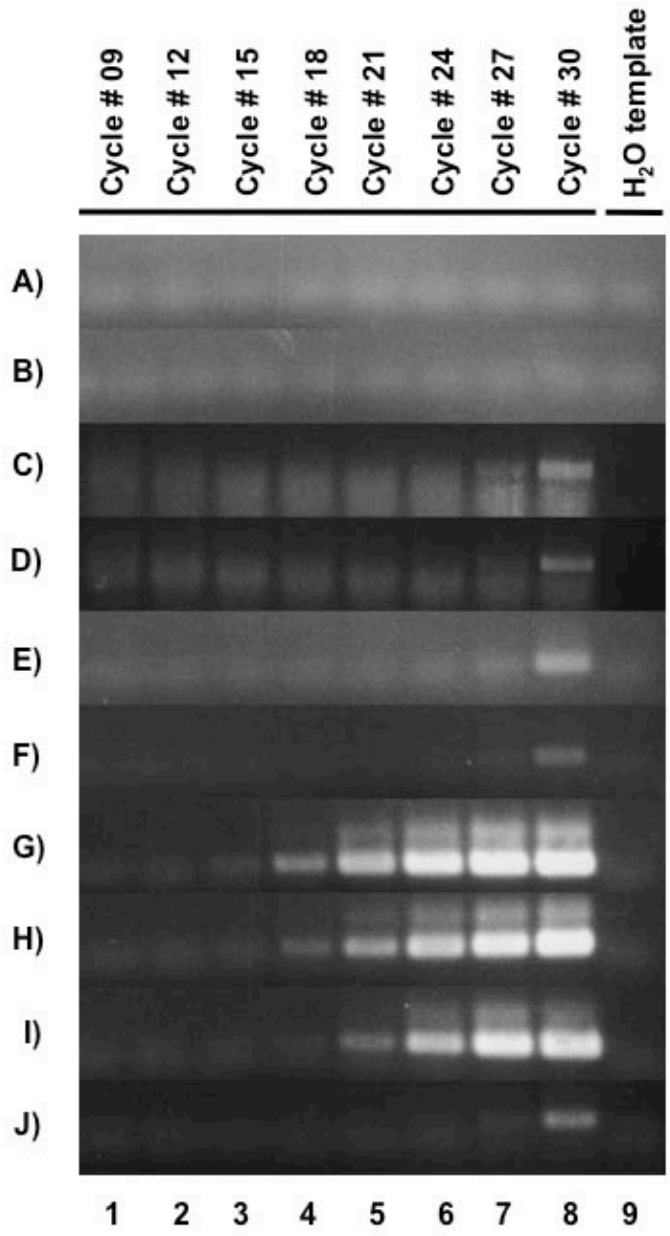


Figure 5.4.2 Agarose gel electrophoresis of DNA fragments obtained from semi-quantitative RT-PCR. Samples were taken every third cycle; a control lacking cDNA as a template was loaded in lane 9. **Panels A and B** : PCR products of reversely transcribed cellular RNA of non transfected cells. **Panels C and D** : PCR products obtained with reversely transcribed cellular RNA of cells transfected with pCMVSRmS. **Panels E and F** : PCR products obtained with reversely transcribed cellular RNA of cells transfected with pCMVSRS. **Panel G** : PCR products obtained with 0.7 µg reversely transcribed SRS-RNA standard. **Panel H** : PCR products obtained with 0.5 µg reversely transcribed SRS-RNA standard. **Panel I** : PCR products obtained with 0.3 µg reversely transcribed SRS-RNA standard. **Panel J** : PCR products obtained with 0.1 µg reversely transcribed SRS-RNA standard.

5.5 Step-wise adaption of StreptoTag-mediated affinity purification for *in vivo* application

As mentioned previously, the final aim of this project is to establish the StreptoTag method for isolation of RNA-binding proteins in physiological conditions. Therefore, a stepwise approach from purely *in vitro*-derived components towards *in vivo* synthesised RNA and protein should be applied.

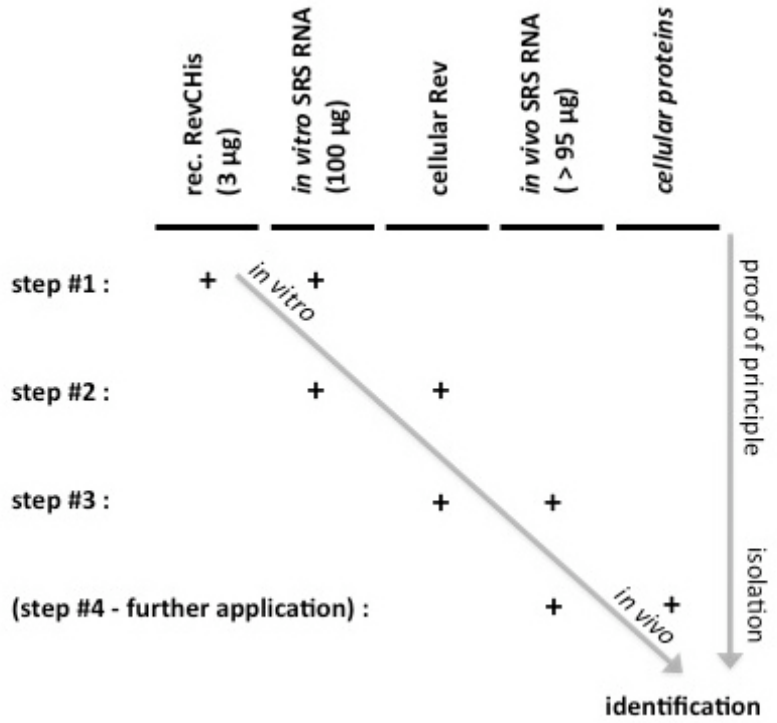


Figure 5.5 Flowchart showing the step-wise approach for establishment of the StreptoTag-mediated affinity purification *in vivo*.

As a first step, proof-of-principle for the StreptoTag method using *in vitro* transcribed SRS-RNA and recombinant Rev protein should be demonstrated. To this aim, 100 µg of *in vitro* transcribed double Strepto-tagged RRE (SRS) RNA was incubated with FPLC-purified recombinant His-tagged HIV-1 Rev protein and the complex loaded onto a streptomycin-coupled sepharose column (see results 5.5.1).

As an intermediate step, recombinant Rev should be substituted by cellular Rev. Therefore, a crude extract of pCMVRev-transfected HEK293 cells was

incubated with *in vitro*-transcribed RNA and the mixture subjected to streptomycin-mediated affinity chromatography (see results 5.5.2).
In the final approach, HEK293 cells should be co-transfected with the SRS-RNA encoding vector and the Rev encoding vector. The RNA-protein interaction is then allowed to occur *in vivo* under biologically relevant conditions. Transfected cells were then subjected to native lysis and subsequently, *in vivo* formed Rev-SRS complexes should be successfully retained on the column (see results 5.5.3). Once this system is established it can be applied to shed light on novel RNA binding proteins under physiological conditions.

5.5.1 Step #1 : Purification of recombinant Rev bound to *in vitro* transcribed SRS-RNA

The first step in establishment of the StreptoTag method for *in vivo* application was to proof the principle of this idea with *in vitro* synthesised components. To this aim, *in vitro* transcribed SRS-RNA was used to retain recombinant Rev on a streptomycin-coupled sepharose column. Prior to incubation with Rev, 100 µg *in vitro* transcribed SRS-RNA were refolded by incubation at 65°C, followed by incubation at 37°C and finally at room temperature for 8 min each. 3 µg of FPLC-purified recombinant RevCHis protein were added, incubated at room temperature for 15 min and diluted in 4 ml of column buffer. The solution was loaded onto the column as described in materials and methods, 4.5.2. Rev protein bound to SRS-RNA should be retained on the column by affinity binding of the StreptoTag to streptomycin, coupled to the sepharose resin. Upon addition of 1 mM streptomycin, the Rev/SRS complex should be eluted and Rev protein be present in the in the respective fraction. The flow-through, five consecutive washing fractions and eluted protein were collected and precipitated by addition of acetone (see materials and methods 4.5.3). The precipitate was dissolved in 1 x protein sample buffer and separated by SDS-PAGE followed by Western blot analysis. Rev was detected using a sheep α-Rev antibody followed by incubation with a mouse anti-sheep secondary antibody conjugated to HRP. The majority of Rev was detected in the flow-

through (Figure 5.5.3, panel A - lane 1) as 3 µg of protein is most probably exceeding the available binding sites on the SRS-RNA. Only the first (Figure 5.5.3, panel A - lane 2) of the five washing steps contained Rev, indicating the strong interaction of protein and SRS-RNA as well as SRS-RNA and column material. Upon addition of 1 mM streptomycin, Rev was successfully eluted and could be detected in the elution fraction (Figure 5.5.3, panel A - lane 7). As a positive control, 0.5 µg of pure recombinant Rev protein was also subjected to analysis and shown to correspond in signal and molecular weight to the respective protein contained in the elution fraction. The difference between the calculated molecular weight of Rev, which is 13 kDa, and the detected molecular weight of 17 kDa is consistent with that of other proteins rich in basic amino acid residues (Nalin, Purcell et al. 1990). To monitor efficacy of precipitation of protein contained in FPLC-fractions, 3 µg of recombinant RevCHis were co-precipitated in each purification experiment (Figure 5.5.3, panel A - lane 9). Here, this signal is hardly visible due to signal interference with recombinant Rev in lane 10.

5.5.2 Step #2 : Purification of cellular Rev bound to *in vitro* transcribed SRS-RNA

As a second, intermediate step in establishment of the StreptoTag method for *in vivo* applications, cellular Rev expressed as a EGFP-Rev fusion protein was isolated by StreptoTag affinity purification using *in vitro* transcribed SRS-RNA. Expression of Rev protein as a fusion with green fluorescent protein was inevitable due to failure of all antibodies tested to recognise cellular HIV-1 Rev protein (data not shown). Antibodies tested included three different lots of polyclonal sheep α-Rev antibody (Fitzgerald) as well as two monoclonal mouse α-Rev antibodies (# 3058 and # 3060) obtained from NIBSC UNaids.
For the purification approach 10^7 HEK293 cells in a 10 cm dish were transfected with 25 µg of the EGFP-Rev encoding vector pEGFPRev (see appendix 8.2) and proteins were isolated after 48 hours by native cell fractionation to ensure a maximum yield of RNA-bound protein (see materials and methods 4.3.6). Nuclear and cytoplasmic fractions were unified and

incubated with 100 µg of refolded *in vitro*-transcribed SRS-RNA for 15 min at room temperature, followed by a stepwise loading of the complexes onto the column (see materials and methods 4.5.2). If cellularly expressed EGFP-Rev would bind to *in vitro*-transcribed SRS-RNA, it was expected to be retained on the column. Elution of the Rev/SRS complexes, protein concentration and SDS-PAGE of fractionated proteins was performed exactly as described in step #1 (see results 5.5.1). EGFP-Rev was detected in different fractions using a mouse α-EGFP antibody as a primary, and a rabbit α-mouse antibody conjugated to HRP as a secondary antibody. In contrast to the previous approach with recombinant Rev protein (see results 5.5.1), 100 µg of *in vitro* transcribed SRS-RNA seem to be sufficient to retain all cellularly expressed EGFP-Rev proteins on the column as no signal is obtained in the flow-through (Figure 5.5.3, panel B - lane 1) and washing step 1 fractions (Figure 5.5.3, panel B - lane 2). However, the interaction of *in vivo* synthesized EGFP-Rev with *in vitro* transcribed SRS-RNA seems to be weaker than with recombinant RevCHis, as some EGFP-Rev protein appears to be eluted during washing steps 2 - 4 (Figure 5.5.3, panel B - lanes 3 - 5). This may be due to the fusion of Rev to EGFP, which increases molecular weight and might reduce affinity of Rev to its target RNA. Nevertheless, the majority of the fusion protein was eluted upon addition of 1 mM streptomycin and could be detected in the elution fraction (Figure 5.5.3, panel B - lane 7). Recombinant RevCHis used as a positive control as well as the precipitated recombinant Rev protein (Figure 5.5.3, panel B - lanes 9 and 10) was not detected, as an α-EGFP antibody was used.

5.5.3 Step #3 : Purification of cellular Rev bound to *in vivo* transcribed SRS-RNA

Having successfully shown that the principle of the StreptoTag method works for *in vitro*-derived components (Figure 5.5.3, panel A), and having isolated EGFP-Rev fusion protein from crude cell extracts in the intermediate step (Figure 5.5.3, panel B), the final approach was to apply the StreptoTag method for purification of *in vivo*-derived components. To this aim, EGFP-Rev

should be isolated from cells co-transfected with the protein and the SRS-RNA encoding vectors. Therefore, 10^7 HEK293 cells in a 10 cm dish were transfected with the plasmids pCMVSRS and pEGFPRev in a ratio of 3 : 1. After 48 hours, nuclear and cytoplasmic proteins were isolated by native cell fractionation (see materials and methods 4.3.6), unified and stepwise loaded onto the column as described in materials and methods, 4.5.2. Here, the protein-RNA interaction was allowed to occur under physiological conditions within the cell. The Rev/SRS complex established in the cellular environment was expected to be retained on the column by affinity binding of the StreptoTag to the streptomycin-coupled sepharose resin. Elution of the Rev/SRS complexes, protein concentration and SDS-PAGE of fractionated samples was performed exactly as described in step #1 (see results 5.5.1). Again, EGFP-Rev was detected in different fractions using a mouse α-EGFP antibody as a primary, and a rabbit α-mouse antibody conjugated to HRP as a secondary antibody. As in 5.5.3, no EGFP-Rev was found in the flow-through (Figure 5.5.3, panel C - lane 1) and washing step 1 fractions (Figure 5.5.3, panel C - lane 2). In the washing steps 2 - 4 (Figure 5.5.3, panel C - lanes 3 - 5) decreasing amounts of EGFP-Rev protein was found and finally no more protein was detected in the last washing fraction (Figure 5.5.3, panel C - lane 6). The majority of EGFP-Rev was successfully eluted and detected in the elution fraction (Figure 5.5.3, panel C - lane 7). As in 5.5.2, recombinant RevCHis protein used as a positive control as well as the precipitated recombinant Rev protein (Figure 5.5.3, panel C - lanes 9 - 10) was not detected, as an α-EGFP antibody was used. Hence, the establishment of the StreptoTag method *in vivo* was successfully shown, subsequently allowing investigations of protein-RNA interactions under physiological conditions.

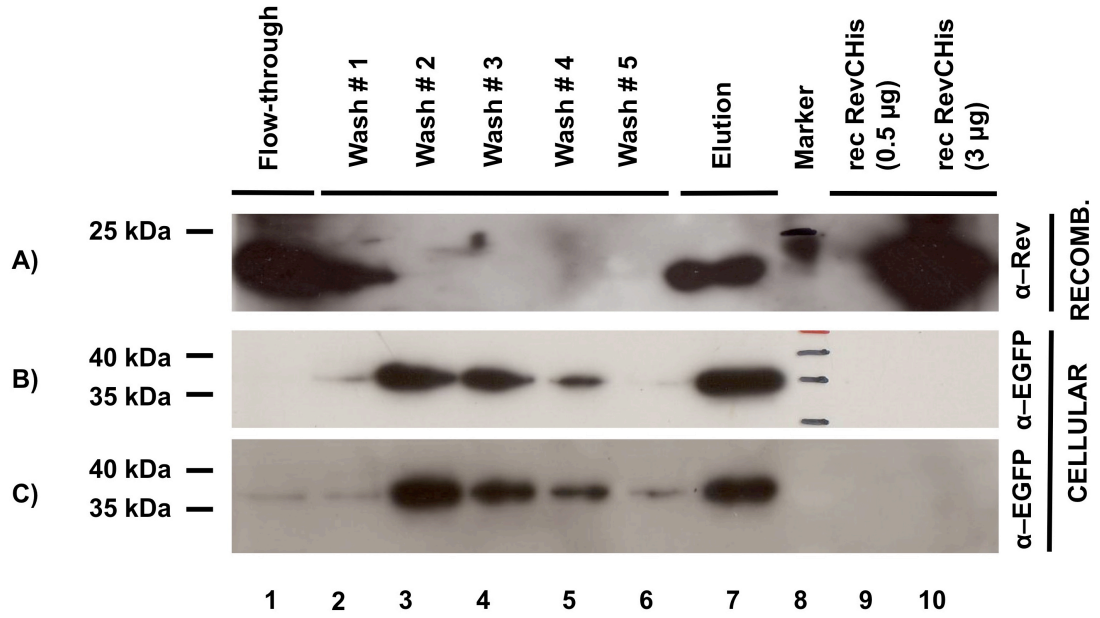


Figure 5.5.3 Stepwise approach for establishment of the StreptoTag method *in vivo*. **Panel A** : Western blot : Purification of recombinant RevCHis using *in vitro* transcribed SRS-RNA. **Panel B** : Western blot : Purification of cellular EGFP-Rev fusion protein using *in vitro* transcribed SRS-RNA. **Panel C** : Western blot : Purification of EGFP-Rev fusion protein from HEK293 cells co-transfected with the RNA and the protein encoding vector. **Lane 1** : flow – through. **Lanes 2-6** : wash fractions. **Lane 7** : eluted protein. **Lane 8** : recombinant His-tagged Rev protein. **Lane 9** : acetone precipitated recombinant Rev protein.

5.5.4 Step #4 : Purification of cellular proteins bound to *in vivo* transcribed-SRS-RNA

As the *in vivo* StreptoTag method is based on RNA-protein interactions in the cellular environment of HEK293 cells, non-specific interference might occur. Cellular proteins may interact with and bind to the transiently transcribed SRS-RNA and therefore block RRE binding sites for EGFP-Rev. This may result in insufficient binding of the specific protein and therefore might reduce amounts to levels beyond detection limits. To investigate the presence of interfering proteins, 10^7 HEK293 cells in a 10 cm dish were transfected with 25 µg of the SRS-RNA encoding vector pCMVSRS only. After 48 hours, nuclear and cytoplasmic proteins were isolated by native cell fractionation (see materials and methods 4.3.6), unified and stepwise loaded onto the column as described in materials and methods, 4.5.2. The transfection of high

amounts of plasmid DNA and respectively presence of vast amounts of target RNA should allow binding of sufficient amounts of potentially interfering endogenous cellular proteins to be detected by silver staining of the respective protein gel. Any protein bound to the SRS-RNA is retained on the column, eluted upon addition of 1 mM streptomycin and should be found in the elution fraction. As in previous experiments, also the flow-through, the consecutive wash and elution fractions were collected and contained proteins precipitated (see materials and methods 4.5.3). The precipitate was dissolved in 1 x protein sample buffer and separated by SDS-PAGE. Silver staining of the gel was performed, as this sensitive method allows detection of even small amounts of any protein contained in the fraction. Regularly, the majority of cellular proteins was found in the flow-through (Figure 5.6.2, panel A - lane 1). Decreasing amounts of proteins were washed from the column during washing steps 1 - 3 (Figure 5.6.2, panel A - lanes 2 - 4) and no protein was found in fractions of washing steps 4 - 5 (Figure 5.6.2, panel A - lanes 5 - 6). No protein of a size that equals that of EGFP-Rev was found in the elution fraction (Figure 5.6.2, panel A - lane 7). However, potentially interfering proteins of different sizes were detected in the elution fraction and further characterised by mass spectrometrical analysis (see results 5.7.1).

5.6 Isolation of cellular MMTV Rem protein

After successful establishment of the StreptoTag method for *in vivo* applications using the well known interaction of HIV-Rev and RRE, the method should be applied to investigate interaction of the MMTV Rem protein with its RNA-binding partner RmRE. As described previously (see results 5.2.2), *in vitro* transcription of full-length SRmS-RNA was not possible. Thus, a stepwise establishment of the StreptoTag-mediated affinity purification as performed with HIV-1 Rev and RRE was not possible. Therefore, purification readily started with the *in vivo* setup.

5.6.1 Purification of cellular Rem bound to *in vivo* transcribed SRmS-RNA

The established *in vivo* StreptoTag method should now be applied to investigate the interaction of MMTV Rem and its responsive element RmRE analogue to the HIV-1 Rev/RRE system. Due to insufficient resources of the α-Rem antibody (M66; Hochman, Park et al. 1990) Rem had to be expressed as EGFP-Rem fusion protein for further detection. EGFP-Rem should be isolated out of HEK293 cells co-transfected with the RNA and the protein encoding vector. Therefore 10^7 HEK293 cells in a 10 cm dish were transfected with the SRmS-RNA encoding vector pCMVSRmS and the EGFP-Rem encoding vector pEGFPRem in a ratio of 3 : 1. After 48 hours, nuclear and cytoplasmic proteins were isolated by native cell fractionation (see materials and methods 4.3.6), unified and loaded stepwise onto the streptomycin-coupled sepharose column as described in materials and methods, 4.5.2. The known interaction of MMTV Rem and RmRE is utilized to bind the protein to the column, mediated by the double Strepto-tagged RmRE-RNA and its affinity to the streptomycin-coupled sepharose. Addition of 1 mM streptomycin should result in elution of bound EGFP-Rem protein. The flow-through, the five consecutive washing fractions and the elution fraction were collected and contained protein precipitated with acetone (see materials and methods 4.5.3). The precipitate was dissolved in 1x protein sample buffer and separated by SDS-PAGE. EGFP-Rem was detected using a mouse α-EGFP antibody (1:1000 - Clontech) as a primary, and a rabbit α-mouse antibody conjugated to HRP as a secondary antibody (1:10000 - DAKO). In contrast to the HIV-1 Rev/RRE system, here EGFP-Rem was exclusively detected in the flow-through (Figure 5.6.1, panels A and B - lane 1) and the first wash fraction (Figure 5.6.1, panels A and B - lane 2). EGFP-Rem was neither detected in the consecutive washing fractions 2 - 5 (Figure 5.6.1, panels A and B - lanes 3 - 6) nor in the elution fraction (Figure 5.6.1, panels A and B - lane 7). As seen from the multiple bands detected in the flow-through fraction, EGFP-Rem appears to be processed either within cells or during preparation of protein extracts. Overall, full-length EGFP-Rem with the calculated size of 68 kDa (EGFP 29 kDa plus Rem 39 kDa) could not be detected. In contrast, the

proteolytically cleaved EGFP-Rem(p14) (molecular weight 43 kDa), which is interacting with SRmS-RNA was found in the flow-through fraction (Figure 5.6.1, panels A and B - lane 1) and the first wash fraction (Figure 5.6.1, panels A and B - lane 2). Two smaller cleavage products of 36 kDa and 30 kDa, respectively, were additionally detected in these fractions (Figure 5.6.1, panels A and B - lane 1). This result was confirmed by SDS-PAGE of the obtained fractions followed by silver-staining of the respective gel. Again, no protein was detected in the elution fraction (Figure 5.6.1, panel C – lane 7)

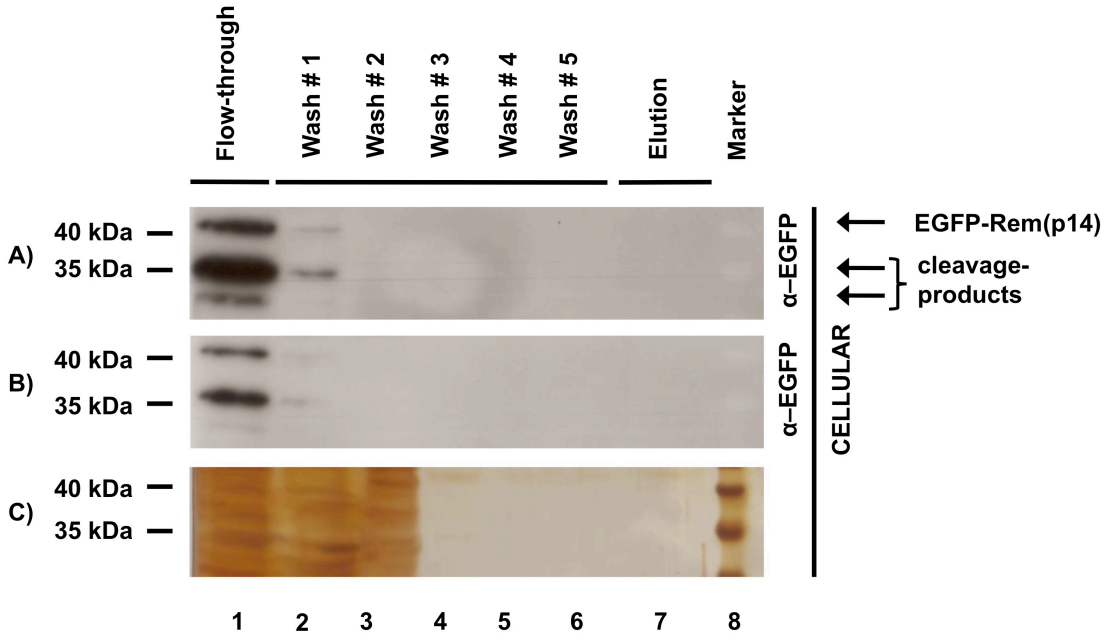


Figure 5.6.1 Approach to isolate MMTV Rem protein applying the StreptoTag method *in vivo*. Panels A and B : Western blot : Affinity purification of cellular EGFP-Rem protein using *in vivo* transcribed SRmS-RNA. **Panel C** : Silver stain : AgNO3-stained SDS-PAGE of protein fractions obtained during purification of cellular EGFP-Rem with *in vivo* transcribed SRmS-RNA.

5.6.2 Purification of cellular proteins bound to *in vivo* transcribed SRmS-RNA

After StreptoTag-mediated affinity purification of cellular EGFP-Rem was not successful, the next step was to determine if endogenously cellular proteins may interact with and bind to the transiently transcribed SRmS-RNA and therefore block RmRE binding sites for EGFP-Rem.

Consequently, levels of SRmS-RNA bound EGFP-Rem(p14) will decrease by lack of sufficient free binding sites and the level of EGFP-Rem(p14) necessary for immunodetection will not be reached. To investigate, if this might have occured, 10^7 HEK293 cells in a 10 cm dish were transfected with 25 µg of the SRmS-RNA encoding vector pCMVSRmS only. After 48 hours, nuclear and cytoplasmic proteins were isolated by a native cell fractionation (see materials and methods 4.3.6), unified and stepwise loaded onto the streptomycin-coupled sepharose column (see materials and methods 4.5.2). This should allow binding of sufficient amounts of interfering endogenous cellular proteins to be detected by silver staining. Any protein bound to the SRmS-RNA is retained on the column, eluted upon addition of 1 mM streptomycin and precipitated from the elution fraction. The flow-through, the five consecutive washing fractions and the elution fraction were collected and precipitated with acetone (see materials and methods 4.5.3). The precipitate was dissolved in 1x protein sample buffer and separated by SDS-PAGE. Silver staining of the gel was performed, as this sensitive method allows detection of any protein in the fraction. The majority of cellular proteins was found in the flow-through (Figure 5.6.2, panel B - lane 1). Decreasingly amounts of proteins were washed away in washing steps 1 - 3 (Figure 5.6.2, panel B - lanes 2 - 4) and no protein was detected in washing steps 4 - 5 (Figure 5.6.2, panel B - lanes 5 - 6). No protein of a size that equals that of EGFP-Rem(p14) is found in the elution fraction (Figure 5.6.2, panel B - lane 7). Potentially interfering proteins of different sizes which were binding to the SRmS-RNA, however, were further characterized by mass spectrometrical analysis (see results 5.7.1).

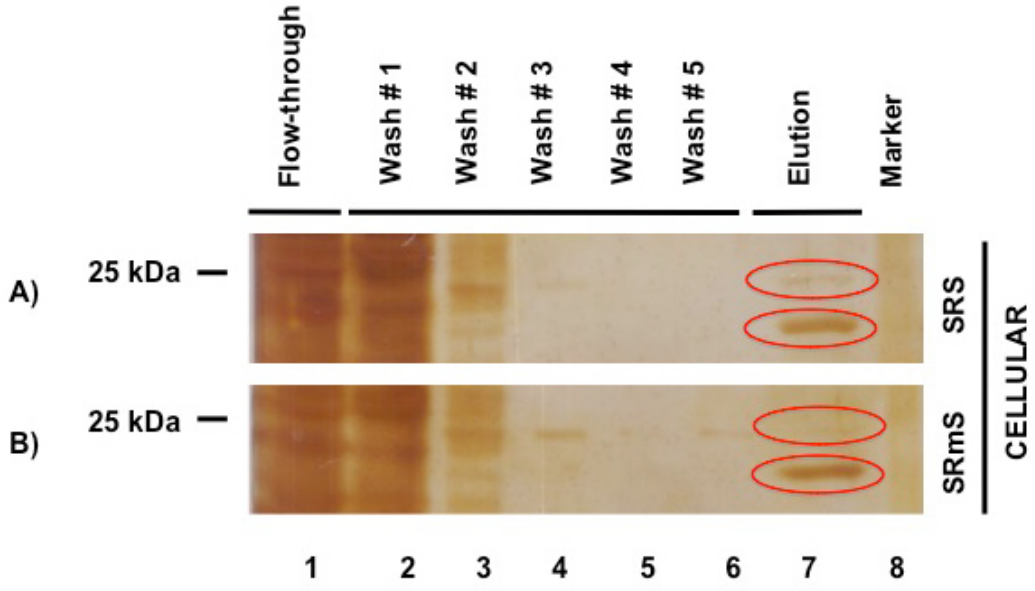


Figure 5.6.2 Isolation of endogenous SRS/SRmS binding proteins by application of the StreptoTag method *in vivo*. Panel A - lane 7 : Affinity purification of HEK293 cells transfected with the SRS encoding vector only. Bands in the elution fraction marked in red were cut out and analyzed by mass spectrometry. Upper band was identified as human glutathione S-transferase and lower band as human dCTP pyrophosphatase 1. **Panel B - lane 7** : Affinity purification of HEK293 cells transfected with the SRmS encoding vector only. Bands in the elution fraction marked in red were cut out and analyzed by mass spectrometry. As in panel B the upper band was identified as human glutathione S-transferase and the lower band as human dCTP pyrophosphatase 1.

5.6.3 Evaluation of functional MMTV Rem/SRmS interaction *in vivo*

Using the StreptoTag method it was not possible to show interaction of Rem protein with the double Strepto-tagged Rem responsive element, the SRmS-RNA. This result might suggest that the Strepto-tags interfere with formation of the secondary structure of the RmRE-RNA. To show, that the 5' and 3' Strepto-tags within the SRmS do not impair binding of Rem, a heterologous HIV-1 gag RNA export assay was performed.

Here, the HIV-1 Gag (see introduction 3.3.1) is expressed under the control of a pCMV promoter. No elements for the export of gag mRNA are included, resulting in its retention in the nucleus. By introducing an export element such as the constitutive transport element (CTE) of Mason-Pfizer monkey virus (MPMV), (Pasquinelli, Ernst et al. 1997; Saavedra, Felber et al. 1997; Yoon,

Lee et al. 1997), downstream of the gag encoding region, the RNA can be recovered and exported by the cellular export machinery (Gruter, Tabernero et al. 1998; Braun, Rohrbach et al. 1999; Bachi, Braun et al. 2000; Jin, Guzik et al. 2003). In contrast, for promoting export of RmRE containing RNA, co-expression of the functional export protein is required. In this system, efficacy of the respective RNA export can be measured by the amount of cytoplasmic HIV p24 Gag protein produced applying Western blotting.

To demonstrate functional interaction of Rem(p14) and its corresponding target sequence the RmRE (p3cRmRE) as well as the Strepto-tagged version SRmS (p3cSRmS) was inserted into the HIV-gag expression construct (Figure 5.6.3.1). As a control, the original HIV-Rev/RRE system was also investigated (p3cRRE). In addition, a construct harbouring the constitutive acting CTE of MPMV was analysed (p3cCTE). A construct lacking any transport element (p3cMCS) served as a negative control.

For analysis, $1.2x10^6$ CrFK cells in one well of a 6-well were transfected with the maximum of 5 µg vector DNA using Lipofectamine 2000 (Invitrogen), (see materials and methods 4.3.5). In co-transfections, the RNA and protein encoding vectors contributed in a ratio of 2 : 1 to the total DNA amount. 48 hours after transfection, cell extracts were prepared and subjected to Western blot analysis. For immunodetection of HIV p24 Gag protein, a monoclonal α-HIV p24 antibody (Polymun) was used.

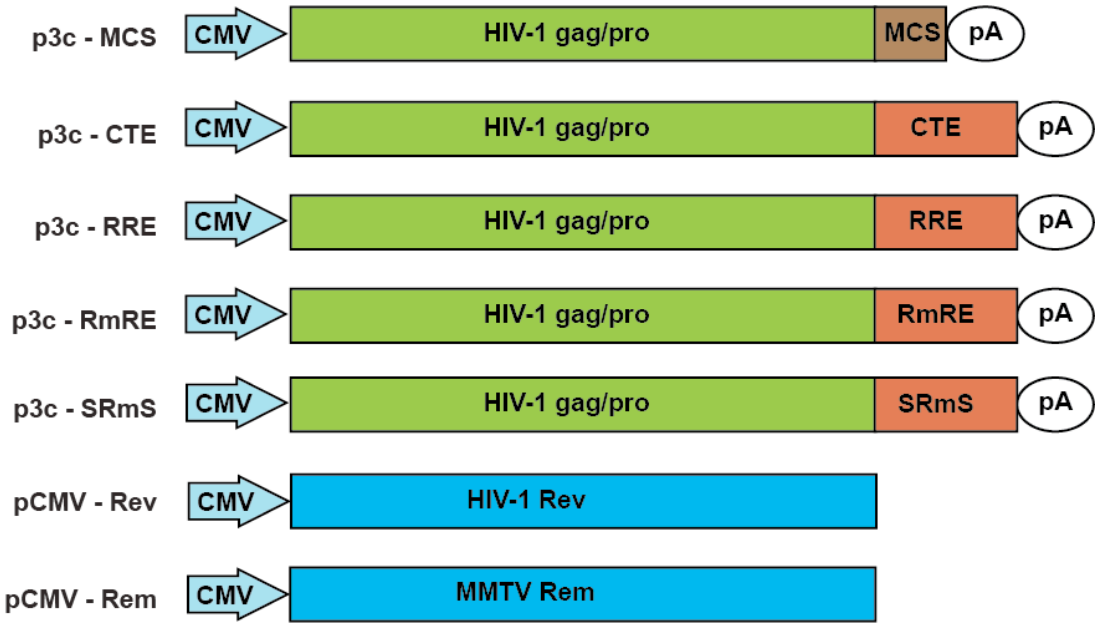


Figure 5.6.3.1 Schematic representation of HIV p24 gag expression constructs used for analysis of functional Rem/RmRE interaction. p3c : back-bone vector; **CMV** : promoter of human Cytomegalovirus; **HIV-1 gag/pro** : RNA encoding p24 gag lacking RNA transport elements; **HIV-1 Rev** : HIV-Rev expression vector; **MMTV Rem** : regulator of export of MMTV mRNA; **MCS** : multiple cloning site; **CTE** : constitutive transport element of Mason-Pfizer monkey virus; **RRE** : HIV-1 Rev responsive element; **RmRE** : MMTV Rem responsive element; **SRmS** : double Strepto-tagged MMTV Rem responsive element; **pA** : poly adenylation signal.

The respective Western blot revealed, that gag mRNA not carrying an export element (p3c-MCS) was retained in the nucleus, hence no p24 Gag protein was detected (Figure 5.6.3.2, lane 2).

Insertion of three copies of an MPMV CTE downstream of the Gag encoding region (p3c-CTE) resulted in recognition and export of the gag-mRNA via a general export pathway (Figure 5.6.3.2, lane 3). The HIV gag-mRNA is processed and p24 Gag protein detected, proofing functional interaction of the CTE export sequence and the corresponding cellular mediator of export.

Replacing the CTE with the HIV RRE as an export element (p3c-RRE) required co-expression of HIV Rev protein, provided by co-transfection of pCMV-Rev (Figure 5.6.3.1). Functional interaction of Rev and its transport element was demonstrated by detection of a robust amount of p24 Gag protein (Figure 5.6.3.2, lane 5).

In contrast, expression of the RRE-containing Gag encoding vector only resulted in retention of the gag-mRNA in the nucleus, thus no p24 was produced (Figure 5.6.3.2, lane 4). Similarly, no Gag protein was detected when extracts of cells were analysed which had been transfected with a construct carrying the MMTV RmRE.

Expression of the RmRE-gag encoding vector only causes gag-mRNA to be retained in the nucleus, as the necessary mediator of export, MMTV Rem, is not present (Figure 5.6.3.2, lane 6). Upon co-expression with Rem, the RmRE harbouring gag-mRNA is recognised by Rem and exported. Accordingly, p24 Gag protein was detected (Figure 5.6.3.2, lane 7).

Finally, the double Strepto-tagged MMTV RmRE, SRmS, was used as an export element. Without co-expression of the corresponding export protein, the gag-mRNA was retained in the nucleus (Figure 5.6.3.2, lane 8). Co-expression of Rem resulted in export of gag-mRNA carrying the SRmS (Figure 5.6.3.2, lane 9), and production of the encoded p24 Gag protein to a similar extent as with constructs containing the wild-type sequence of RmRE (Figure 5.6.3.2, compare lanes 7 and 9).

These results convincingly demonstrate that MMTV Rem protein is functionally expressed in transfected cells and interacts efficiently with its RNA target sequence, the original as well as the Strepto-tagged form. This indicates that the StreptoTag modification does not interfere with formation of RmRE secondary structure, which is essential for its recognition by Rem.

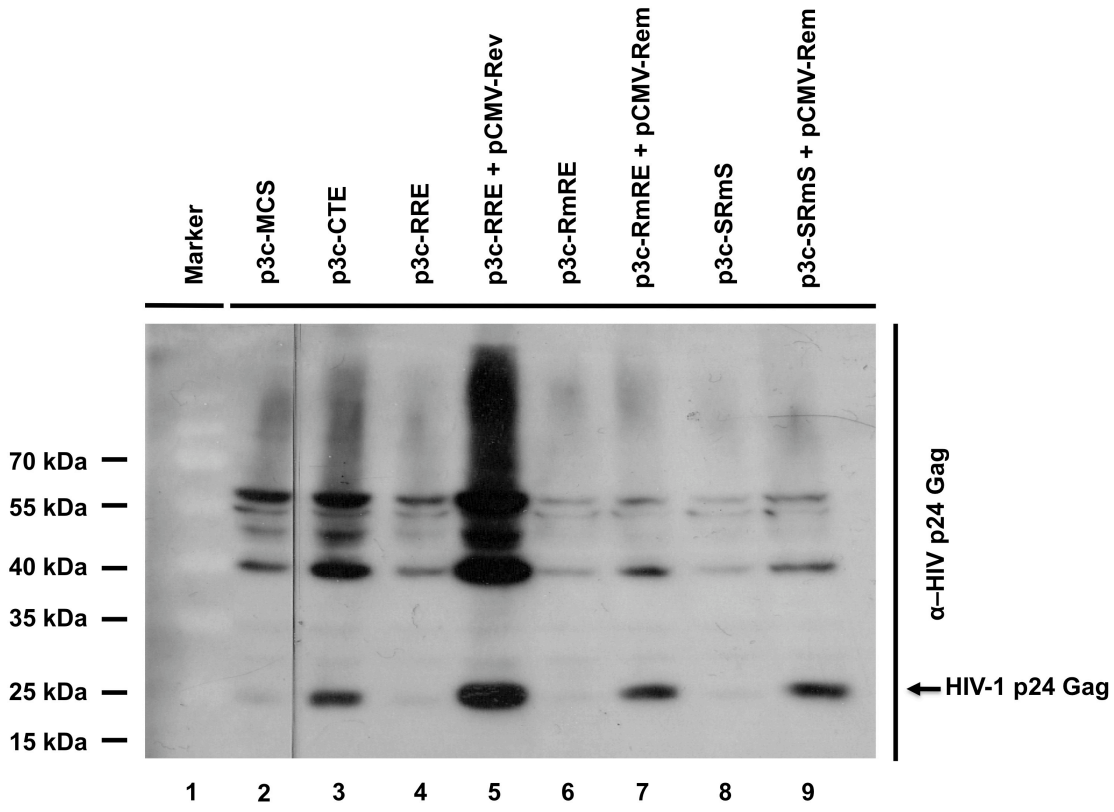


Figure 5.6.3.2 Heterologous HIV-1 gag export assay. Lane 1 : Marker. **Lane 2 :** Cell lysate of CrFK cells transfected with the HIV Gag encoding vector lacking a CTE. **Lane 3 :** Cell lysate of CrFK cells transfected with the HIV Gag encoding vector containing the MPMV CTE. **Lane 4 :** Cell lysate of CrFK cells transfected with the HIV Gag encoding vector containing the HIV-1 RRE. **Lane 5 :** Cell lysate of CrFK cells co-transfected with the HIV Gag encoding vector containing the HIV-1 RRE, and pCMV-Rev expression vector. **Lane 6 :** Cell lysate of CrFK cells transfected with the HIV Gag encoding vector containing the MMTV RmRE. **Lane 7 :** Cell lysate of CrFK cells co-transfected with the HIV Gag encoding vector containing the MMTV RmRE, and pCMV-Rem expression vector. **Lane 8 :** Cell lysate of CrFK cells transfected with the HIV Gag encoding vector containing the double Strepto-tagged MMTV RmRE. **Lane 9 :** Cell lysate of CrFK cells co-transfected with the HIV Gag encoding vector containing the double Strepto-tagged MMTV RmRE, and the pCMV-Rem encoding vector. Equal amounts of total protein were separated by SDS-PAGE and finally subjected to Western-blot analysis using a p24-Gag specific antibody.

5.6.4 Evaluation of functional interaction of EGFP-Rem fusion protein and Strepto-tagged target RNA *in vivo*

As pointed out previously, HIV-1 Rev as well as MMTV Rem are mediating the export of unspliced viral RNA from the nucleus to the cytoplasm. As

demonstrated before (see results 5.6.3), Rev as well as Rem functionally bind to the respective Strepto-tagged target sequence, thus facilitating export of HIV-gag RNA. However, in these experiments the natural viral proteins were investigated, while affinity-chromatographic purification has been performed with MMTV-EGFP-Rem fusion protein. The fluorescent activity of EGFP was utilised in the next experiment aiming at demonstrating functional protein/RNA interaction at the respective fusion protein *in vivo*.

Therefore, CrFK cells were transfected in various combinations with fusion protein, RNA and mock encoding vectors (Figure 5.6.4.1 and Figure 5.6.4.2). Co-transfections with the RNA and protein encoding vectors were carried out in a plasmid ratio of 3 : 1. By transfecting the protein encoding vector only or the protein and mock encoding vectors, fluorescence was expected to be limited to the nucleus. Upon co-transfection of the protein encoding vector and the plasmid, encoding the responsive element bearing RNA, a shift of fluorescence from the nucleus to the cytoplasm should be observed. Visual analysis was carried out using a UV-fluorescence microscope with a specific filter detecting the green fluorescent protein (Axiovert 200 imaging system, ZEISS).

For detection of EGFP-Rev/RRE interactions, 10^4 CrFK cells were transfected with 5 µg of vector DNA. Analysis by fluorescence microscopy was carried out 48 hours post transfection. For comparison, cells were transfected with the EGFP-encoding vector pCMVEGFP only (Figure 5.6.4.1, panel A). In these cells, EGFP was shown to be equally distributed within the cytoplasm and the nucleus (Figure 5.6.4.1, panel A - lane 2). Cells that had been transfected with the EGFP-Rev encoding vector pEGFPRev (Figure 5.6.4.1, panel B), fluorescence was strictly limited to the nucleus (Figure 5.6.4.1, panel B - lane 2). As Rev harbours an N-terminal nuclear localisation sequence (NLS), it is immediately translocated to the nucleus after translation at the endoplasmic reticulum (ER). In contrast, cells co-transfected with the EGFP-Rev encoding pEGFPRev and the RRE encoding vector p3C-RRE (Figure 5.6.4.1, panel C) show fluorescence only within the cytoplasm. Here, translocation occurs as EGFP-Rev located in the nucleus encounters RRE-harbouring RNA, binds to it and transports RNA to the cytoplasm, where it is consequently detected (Figure 5.6.4.1, panel C - lane 2). To investigate if the fusion protein would

also bind to double Strepto-tagged RRE, SRS, cells were co-transfected with the EGFP-Rev encoding pEGFPRev and the SRS encoding vector pCMVSRS (Figure 5.6.4.1, panel D). Exactly as in Figure 5.6.4.1 panel C, EGFP-Rev was detected exclusively in the cytoplasm (Figure 5.6.4.1, panel D - lane 2), demonstrating EGFP-Rev mediated RNA export, associated with protein translocation. The export of the EGFP-Rev/RNA complex occurs only if the RNA carries the responsive element. This was demonstrated in cells co-transfected with the EGFP-Rev encoding vector pEGFPRev and a vector encoding for a mock RNA derived from pcDNA3.1 (Figure 5.6.4.1, panel E). Here, fluorescence was accordingly strictly limited to the nucleus (Figure 5.6.4.1, panel E - lane 2) .

Similar to this experimental setup, cells were also transfected with the corresponding plasmids allowing visual evaluation of EGFP-Rem and SRmS RNA interaction.

As a control, cells were again transfected with the EGFP encoding vector pCMVEGFP only (Figure 5.6.4.2, panel A). Cells transfected with the EGFP-Rem encoding vector pEGFPRem (Figure 5.6.4.2, panel B - lane 2) show a fluorescence strictly limited to the nucleus. In contrast to cells transfected with pEGFPRev (Figure 5.6.4.1, panel B - lane 2), here fluorescence is also visible in little dots surrounding the nucleus (Figure 5.6.4.2, panel B - lane 2). Identical to HIV-1 Rev, Rem harbours an N-terminal NLS, hence it is immediately translocated to the nucleus after translation at the endoplasmic reticulum. As no vector encoding for the RmRE was co-transfected, EGFP-Rem remains in the nucleus and no translocation occurs. In contrast to the HIV-1 Rev/RRE system, where fluorescence is strictly limited to the cytoplasm upon co-transfection of EGFPRev and RRE/SRS encoding vectors (Figure 5.6.4.1, panels C and D - lane 2), in cells expressing EGFP-Rem (pEGFPRem) and RmRE (p3CRmRE) a fluorescent shift to the cytoplasm is observed but still some protein is contained in the nucleus (Figure 5.6.4.2, panel C - lane 2). Co-transfection of vectors encoding EGFP-Rem (pEGFPRem) and the double Strepto-tagged RmRE, SRmS (pCMVSRmS), reveals the same fluorescence pattern (Figure 5.6.4.2, panel D - lane 2) as observed by co-transfecting the non-tagged RmRE (compare Figure 5.6.4.2, panel C - lane 2). In contrast, EGFP-Rem encoding vector (pEGFPRem) co-

transfected with a vector encoding for a mock RNA (pCDNA3.1) as a control resulted in the same fluorescence pattern as transfection with pEGFPRem only (Figure 5.6.4.2, panel E - lane 2). Hence, as shown before (Figure 5.6.3.2, lane 9) expression of the RmRE as double Strepto-tagged hybrid RNA does not impair the export mechanism. Moreover, functional interaction of both target RNAs with the EGFP-fusion protein was clearly demonstrated.

In conclusion, it was demonstrated that MMTV Rem protein in its natural form as well as in fusion with green fluorescent protein functionally interacts with Strepto-tagged RNA and mediates RNA export as shown previously for the Rev/RRE system.

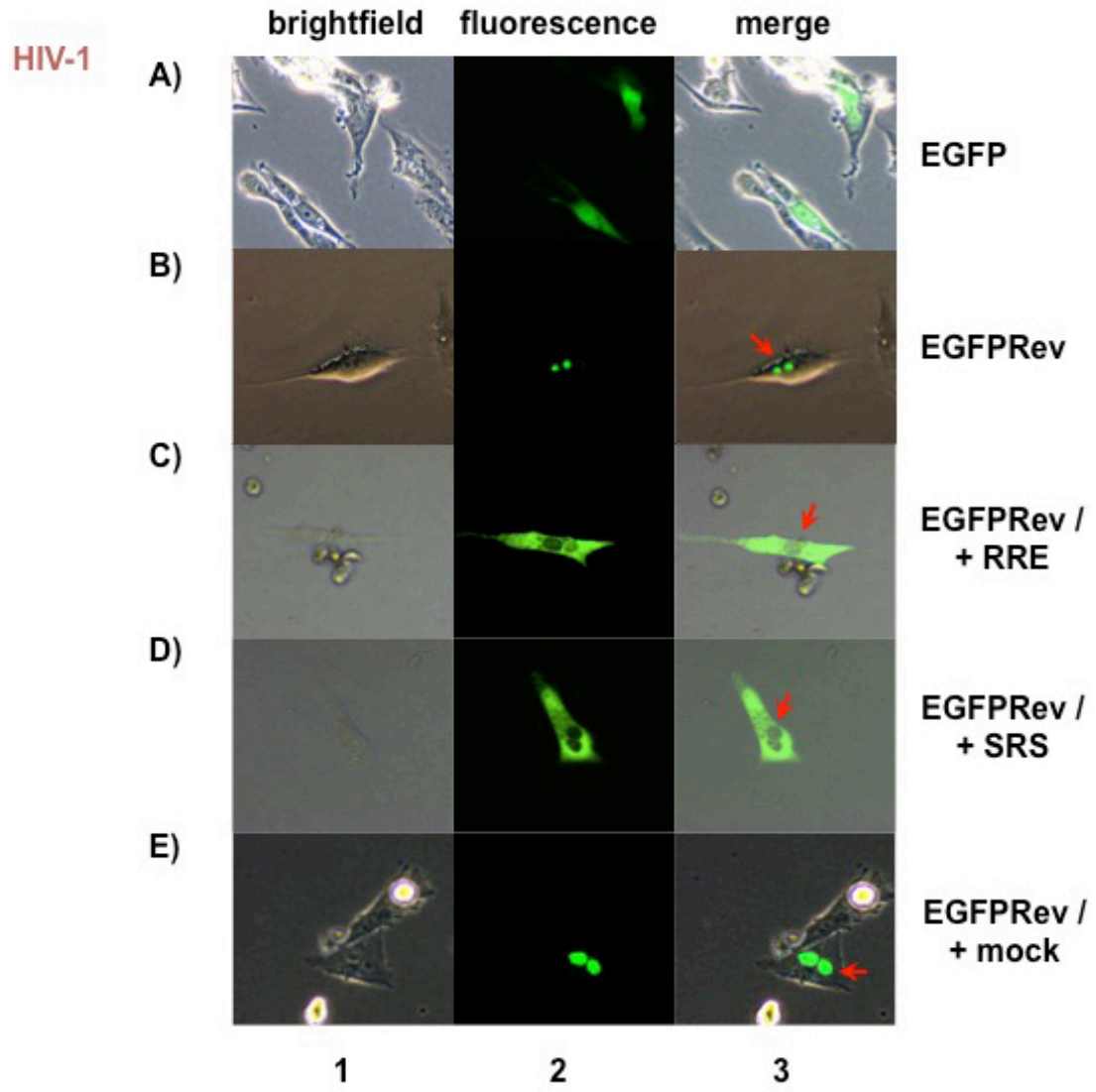


Figure 5.6.4.1 Fluorescence microscopy of CrFK cells transfected with : A) pCMVEGFP vector encoding green fluorescent protein. **B)** pEGFPRev vector encoding fusion protein EGFP-Rev. **C)** pEGFPRev and p3C-RRE vectors encoding fusion protein EGFP-Rev and RRE-RNA. **D)** pEGFPRev and pCMVSRS vectors encoding fusion protein EGFP-Rev and double Strepto-tagged RRE-RNA SRS. **E)** pEGFPRev and pcDNA3.1 vectors encoding fusion protein EGFP-Rev and mock RNA. Representative pictures of a culture with about 40% transfected cells are shown. (Axiovert 200 imaging system, ZEISS; magnification 400x).

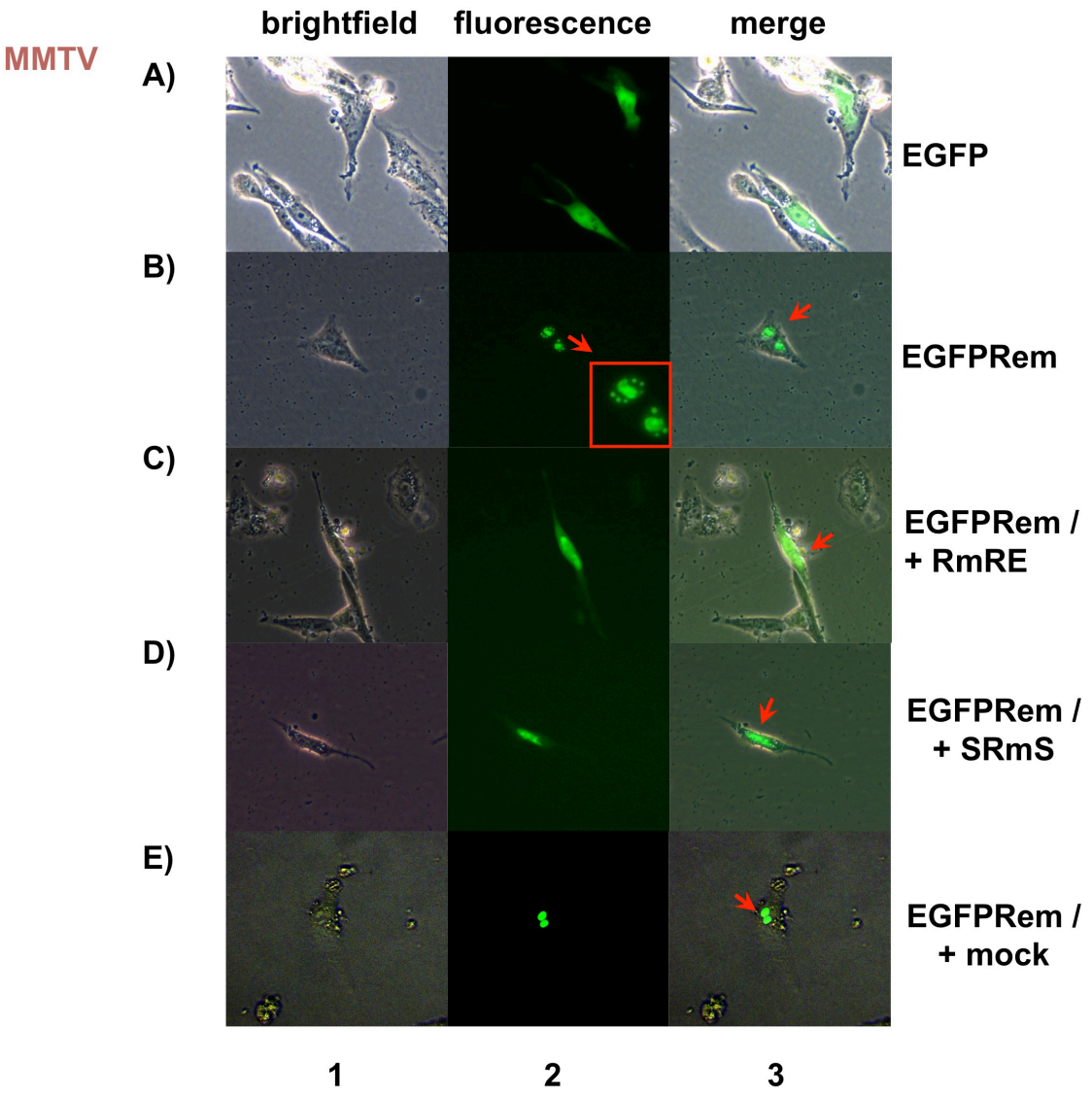


Figure 5.6.4.2 CrFK cells transfected with : A) pCMVEGFP vector encoding green fluorescent protein. **B)** pEGFPRem vector encoding fusion protein EGFP-Rem. **C)** pEGFPRem and p3CEnvLTR-Jun vectors encoding fusion protein EGFP-Rem and RmRE-RNA. **D)** pEGFPRem and pCMVSRmS vectors encoding fusion protein EGFP-Rem and double Strepto-tagged RmRE-RNA SRmS. **E)** pEGFPRem and pcDNA3.1 vectors encoding fusion protein EGFP-Rem and mock RNA. Representative pictures of a culture with about 40% transfected cells are shown. (Axiovert 200 imaging system, ZEISS; magnification 400x).

5.7 Isolation of novel RNA binding proteins

Having shown with the Rev/RRE system that the StreptoTag method can be applied to isolate RNA-binding proteins from eukaryotic cells, a final approach was made to isolate novel putative RNA-binding proteins under physiological conditions.

For isolation of novel RNA interacting proteins any putative target RNA sequence may be cloned and expressed as a double Strepto-tagged RNA hybrid. For identification of endogenous RNA-binding proteins, cells may then be transfected with this RNA encoding vector. Any Strepto-tagged RNA expressed within cells can be isolated with the potentially interacting protein bound and finally purified by affinity chromatography using a streptomycin-coupled sepharose column. In this last approach, cells were transfected with SRS- or SRmS-RNA encoding vectors (Figure 5.7.1.1, panel B), respectively, followed by Strepto-Tag affinity chromatography and analysis of isolated proteins by mass spectrometry.

5.7.1 Screening of HEK293 cells for protein interactions with SRS- and SRmS-RNA

In this approach, HEK293 cells were transfected with either the SRS or the SRmS encoding vector. Interaction of endogenous, potentially RNA binding proteins was allowed to occur in the cellular environment. Complexes formed *in vivo* with novel binding partners were then purified via a streptomycin-coupled sepharose column (see materials and methods 4.5.2) and isolated proteins identified by mass spectrometry (see materials and methods 4.8).

In detail, 10^7 HEK293 cells in a 10 cm dish were transfected with 25 µg of either the SRS or the SRmS encoding vectors, pCMVSRS and pCMVSRmS, respectively. After 48 hours nuclear and cytoplasmic proteins were isolated by native cellular fractionation (see materials and methods 4.8.6), unified and stepwise loaded onto a column as described previously. The flow-through, the five washing fractions and the elution fractions were collected and protein

precipitated by addition of acetone (see materials and methods 4.5.3). The pellet was dissolved in 1 x protein sample buffer and separated by SDS-PAGE. Silver staining of the gel, compatible with subsequent mass spectrometric analysis was performed. As shown in Figure 5.7.1.1, expressing either SRS- or SRmS-RNA, both led to the same results : two distinct proteins of a molecular weight of about 20 kDa and 25 kDa (red circles in Figure 5.7.1.1, panels A and B) were purified. The purifications were carried out in duplicates, the respective protein-bands excised and pooled to increase the amounts subjected to mass spectrometrical analysis. Results identified the larger protein in the elution fraction to be human glutathione S-transferase with a molecular weight of 23.3 kDa (Figure 5.7.1.2) and the smaller protein to be human dCTP pyrophosphatase 1, with a molecular weight of 18.7 kDa (Figure 5.7.1.3).

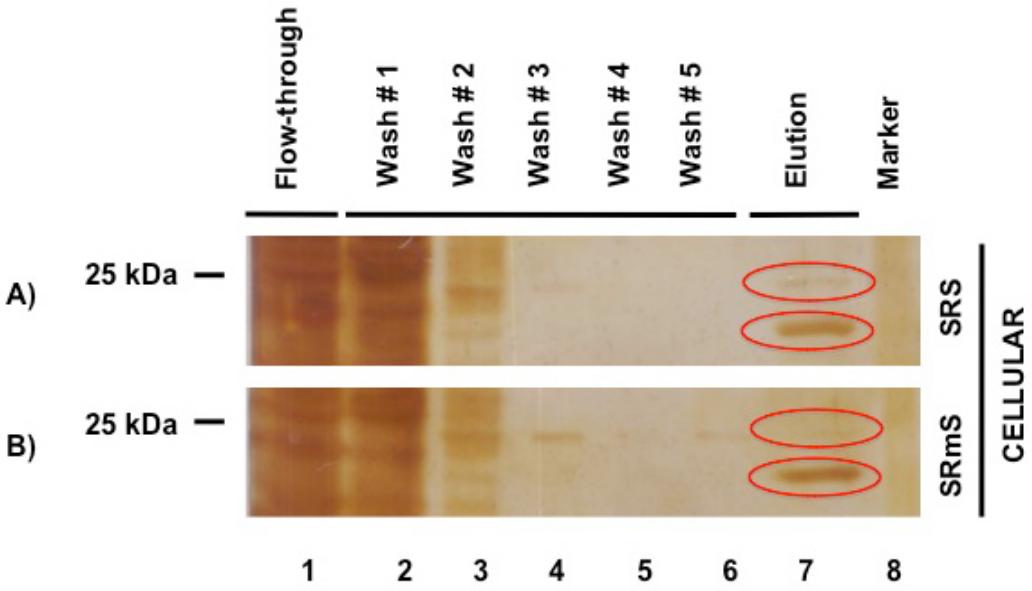
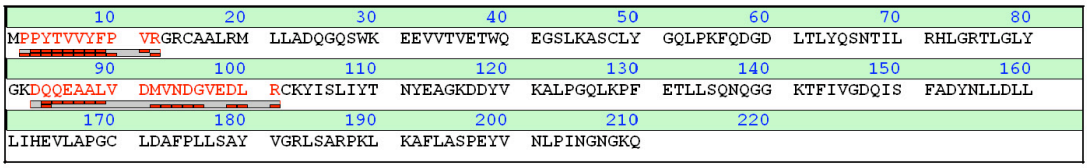


Figure 5.7.1.1 Isolation of endogenous SRS/SRmS binding proteins by application of the StreptoTag method *in vivo*. Panel A - lane 7 : Affinity purification of HEK293 cells transfected with the SRS encoding vector only. Bands in the elution fraction marked in red were cut out and analyzed by mass spectrometry. **Panel B - lane 7** : Affinity purification of HEK293 cells transfected with the SRmS encoding vector only. Bands in the elution fraction marked in red were cut out and analysed by mass spectrometry.

Protein 1: **Glutathione S-transferase P OS=Homo sapiens GN=GSTP1 PE=1 SV=2**

Accession:	GSTP1_HUMAN	**Score:**	161.5
Database:	SwissProt(SwissProt_57.15.fasta)	**MW [kDa]:**	23.3
Database Date:	2010-04-28	**pI:**	5.3
		Sequence Coverage [%]:	14.3
		No. of unique Peptides:	2

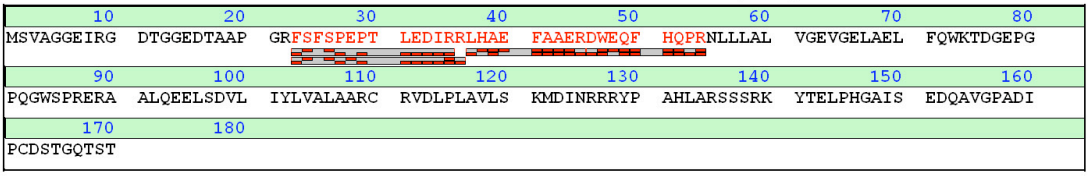


Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
	1	1337.6921	-24.77	1		87.1	0	2-12	M.PPYTVVYFPVR.G	
	1	2116.9512	-14.33	1		74.3	0	83-101	K.DQQEAALVDMVNDGVEDLR.C	

Figure 5.7.1.2 Mass spectrometric analysis MALDI-TOF of pooled upper bands shown in Figure 5.7.1.1, panels A and B. The protein was identified as human glutathione S-transferase P (MW 23.3 kDa) with a significant score of 161.5 whereupon the level of significance is 80.

Protein 1: **dCTP pyrophosphatase 1 OS=Homo sapiens GN=DCTPP1 PE=1 SV=1**

Accession:	DCTP1_HUMAN	**Score:**	235.9
Database:	SwissProt(SwissProt_57.15.fasta)	**MW [kDa]:**	18.7
Database Date:	2010-04-28	**pI:**	4.8
		Sequence Coverage [%]:	18.8
		No. of unique Peptides:	4



Cmpd.	No. of Cmpds.	m/z meas.	Δ m/z [ppm]	z	Rt [min]	Score	P	Range	Sequence	Modification
	1	1537.7993	29.91	1		59.1	0	23-35	R.FSFSPEPTLEDIR.R	
	1	1693.9291	44.09	1		45.2	1	23-36	R.FSFSPEPTLEDIRR.L	
	1	1043.5487	20.92	1		55.9	0	37-45	R.LHAEFAAER.D	
	1	1242.5861	16.94	1		75.7	0	46-54	R.DWEQFHQPR.N	

Figure 5.7.1.3 Mass spectrometric analysis MALDI-TOF of pooled lower bands in 5.7.1.1, panels A and B. The signal was identified as human dCTP pyrophosphatase 1 (MW 18.7 kDa) with a significant score of 235.9 whereupon the level of significance is 80.

6. Discussion

Within the present work, the StreptoTag-mediated purification of RNA-binding proteins has been successfully established for *in vivo* applications. This provides an interesting new tool for investigating potential RNA-protein interactions under physiological conditions. In the course of its development, the StreptoTag method has been optimised, i.e. by modification of the RNA-aptamer (Dangerfield, Windbichler et al. 2006) or addition of a second StreptoTag at the 5' end of the target RNA (Müllner Matthias, Diploma Thesis 2007). The adaption of the StreptoTag method from using only two simple *in vitro* components, namely the *in vitro* transcribed RNA and a recombinant protein, to the StreptoTag method *in vivo*, as presented in this work, also implied dealing with the many times more complex environment of the cell. In this regard, a step-wise approach for establishment of the method *in vivo* based on the HIV-1 Rev/RRE system was a necessary and successful approach. In contrast, the first application of the StreptoTag method *in vivo*, investigating the RNA-protein interaction of MMTV Rem and RmRE, was encountering obstacles *in vitro* as well as on the cellular level both of which could not be overcome so far.

The step-wise approach for the establishment of the StreptoTag method *in vivo*, as performed for the HIV-1 Rev/RRE system, could not be transferred and applied to the MMTV Rem/RmRE system, as *in vitro* transcription of double Strepto-tagged RNA was impaired and resulted in incomplete transcripts. The reason for incomplete transcription may be due to the sequence of the SRmS (Figure 6.1). 200 nucleotides after transcription initiation at the T7 promoter, the T7 polymerase encounters a 12 base pair stretch of adenosines located within the RmRE, intercepted by only one guanine. The 12 adenosines are most likely weakening the 8 base pair DNA-RNA formed by the T7 polymerase during RNA synthesis thereby inducing dissociation of the DNA-RNA-polymerase complex which results in incomplete transcripts. Nevertheless, RNA containing a wild-type MMTV RmRE (nt 1-496) which also contains the adenosine rich sequence was previously successfully transcribed *in vitro* using the T7 polymerase (Mertz, Chadee et

al. 2009). In contrast, *in vivo* transcription by cellular polymerases was found to overcome this obstacle as shown by full-length RNA synthesized from transfected pCMVSRmS plasmid (Figure 5.4.2, panels C and D - lane 8).

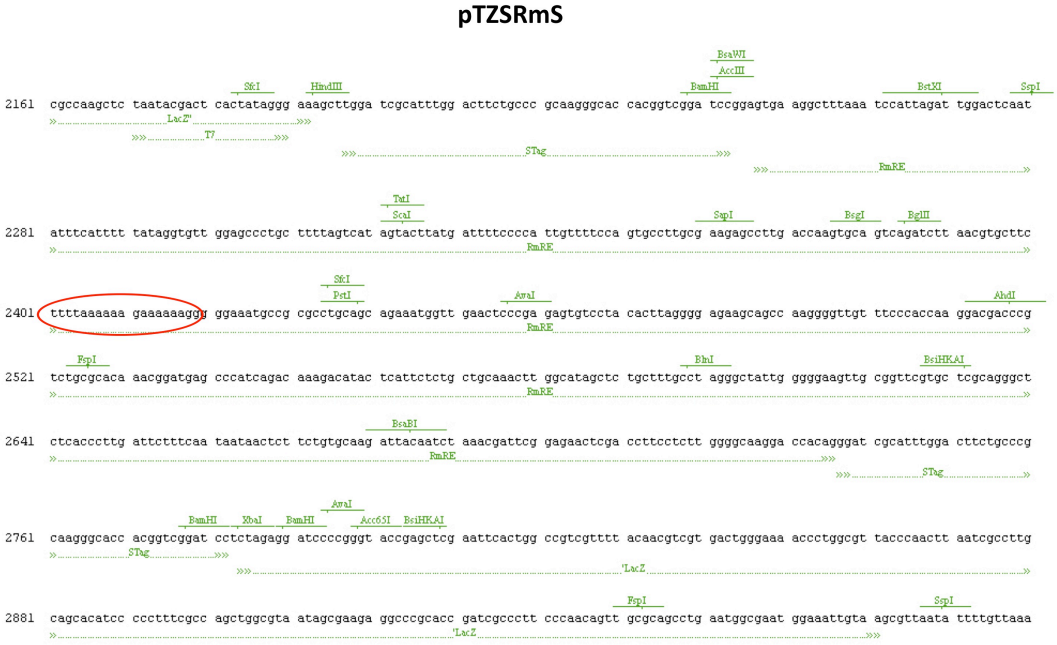


Figure 6.1 Partial sequence of the pTZSRmS encoding vector used for *in vitro* transcription of SRmS RNA. T7, promoter recognized by T7 polymerase; **STag,** StreptoTag**; RmRE,** MMTV Rem responsive element; **LacZ**, gene coding for enzyme β-galactosidase**.**

However, although target RNA *in vivo* has been synthesized, in contrast to purification of HIV-Rev, MMTV Rem(p14) could not be isolated from HEK293 cells co-transfected with the Rem(p14) protein- and SRmS RNA-encoding vector (Figure 5.6.1). Rem had to be expressed as an EGFP fusion protein to facilitate detection due to insufficient resources of α-Rem antibody (M66). It was shown that EGFP- fusion neither with Rev nor with Rem inhibits RNA interaction *in vivo* (see results 5.6.4; and Stauber, Gaitanaris et al. 1995).
Nevertheless, the multiple possibilities of interference on the cellular level e.g. protease-mediated degradation have to be considered when interaction of the Rem protein and the SRmS-RNA occurs under physiological conditions. As shown in Figure 5.6.1, application of the StreptoTag method *in vivo* for affinity purification of EGFP-Rem fusion protein did not result in isolation of any

specific binding protein present in the elution fraction. Instead, multiple proteins of different sizes ranging from 30 kDa to 43 kDa were found in the flow-through and wash 1 fraction. Full-length EGFP-Rem was not detected in any of the fractions in contrast to its cleavage product EGFP-Rem(p14). This indicates that proteolytical processing occurs within HEK293 cells cleaving EGFP-Rem (68 kDa) into EGFP-Rem(p14) (43 kDa) and gp32Rem, and into further fragments of 30 kDa and 36 kDa, respectively. This might reduce levels of EGFP-Rem and EGFP-Rem(p14) below detection limits in Western blot analysis using the α-EGFP antibody. Thus, at this point it is not possible to determine whether EGFP-Rem or EGFP-Rem(p14) might have bound to the Strepto-tagged RNA and subsequently were purified by affinity chromatography.

Although proteolytic-cleavage might have been the main cause for a failure of detection, at this point also the second part of the purification system, the Strepto-tagged target RNA, SRmS was analysed for its integrity.

The StreptoTag-mediated affinity purification was successfully established for the HIV-1 Rev/RRE system and EGFP-Rev was isolated from co-transfected HEK293 cells (see results 5.5.3). So far, the Strepto-tags added to the target RNA were not considered to impair the binding of the protein. This had to be verified also for the Rem/SRmS interaction. To show that the 5' and 3' Strepto-tags within the SRmS do not impair the binding of Rem, a heterologous HIV-1 gag RNA export assay was performed (see results 5.6.3). As shown in Figure 5.6.3.2 - lane 9, HIV-1 gag mRNA carrying the double Strepto-tagged MMTV RmRE is effectively exported by the co-expressed MMTV Rem protein and consequently p24 protein was expressed, indicating that the Strepto-tags did not impair formation of the RmRE secondary structure essential for its recognition by Rem.

Additional to the cleavage of EGFP-Rem by endogenous peptidases, the specificity of the RNA-protein interaction of EGFP-Rem and SRmS in comparison to the highly specific EGFP-Rev and SRS interaction (Iwai, Pritchard et al. 1992; Mann, Mikaelian et al. 1994; Wang, Ma et al. 2010) might be considered as a factor decreasing detection probability of purified Rem in this approach.

A hint for only modest binding of Rem to its target RNA might be drawn from fluorescence microscopy data (Figure 5.6.4.2). Both proteins, Rev and Rem, carry an N-terminal NLS (Cullen, Hauber et al. 1988; Stauber, Gaitanaris et al. 1995; Stauber, Afonina et al. 1998; Indik, Günzburg et al. 2005) which in absence of the respective RNA target sequence restricts the protein to the nucleus (Kalland, Szilvay et al. 1994; Richard, Iacampo et al. 1994; D'Agostino, Ciminale et al. 1995). Upon co-transfection of SRS, the vast amount of Rev translocated into the cytoplasm as shown by evenly distributed fluorescence sparing the nuclei (Figure 5.6.4.1, panel D - lane 2). This clear-cut picture was not seen with MMTV Rem interacting with RmRE- or SRmS-RNA. Here, only weak fluorescence was found in the cytoplasm and nuclei still appeared to contain substantial amounts of the EGFP-Rem fusion protein (Figure 5.6.4.2, panel D - lane 2). Thus, the less effective translocation observed here is in contrast to the interaction of EGFP-Rev and RRE/SRS, possibly indicating the reduced specificity of the MMTV Rem/RmRE interaction.

Taking this into account, also interference of cellular proteins potentially binding to the SRmS target RNA might hinder effective isolation of Rem. In expression, besides the co-expressed protein of interest, target RNA within the cell may also be covered by many endogenous cellular proteins due to unspecific interactions which are thereby blocking free binding sites for the RNA-binding protein of interest. StreptoTag-mediated affinity purification of protein extracts derived from HEK293 cells transfected with the SRS- or SRmS-RNA encoding vector only resulted in purification of two unspecific interacting endogenous proteins. Further characterisation identified these proteins as human glutathione S-transferase P and human dCTP pyrophosphatase 1 (Figure 5.7.1.1, panels A and B - lane 7).

According to the ExPASy proteomics server (Swiss Institute of Bioinformatics) glutathione S-transferase (Mannervik, Alin et al. 1985; Kano, Sakai et al. 1987) as well as dCTP pyrophosphatase do not carry any DNA/RNA binding motifs. However, as the same unspecific proteins were purified with both double Strepto-tagged RNA target sequences, the HIV-1 RRE as well as the MMTV RmRE (Figure 5.7.1.1, panels A and B - lane 7), which differ in sequence as well as secondary structure, this unspecific binding might be

rather due to the presence of the Strepto-tag itself. This tags show the same secondary structure in both RNAs and therefore might bind proteins independent of the respective target sequence.

The StreptoTag method *in vivo*, as presented in this work, was the first application of the StreptoTag affinity purification in a cellular environment and so far no other work has been published indicating a possible interaction of the StreptoTag RNA aptamer with cellular proteins. The low specificity of the system encountered during purification of Rem might however be overcome by applying less stringent conditions, such as increasing incubation by e.g. incubating the RNA-protein mixture with the sepharose over night.

The problem of non-specific binding has also been described for other RNA aptamer affinity tags, such as those based on the interaction with streptavidin and dextran 512, both of which also allow elution of bound proteins under native conditions (Srisawat and Engelke 2001).

Besides the incorporation of an artificially selected RNA motif, such as the StreptoTag (Bachler, Schroeder et al. 1999; Dangerfield, Windbichler et al. 2006), Sephadex RNA motif and Streptavidin RNA motif (Srisawat and Engelke 2001; Srisawat, Goldstein et al. 2001), different alternative methods for tagging a specific RNA are available, all of them implying several obstacles.

Thus, chemical tagging of RNA during *in vitro* transcription with e.g. biotin, fluorescent dyes or digoxeginin, leads to structural variations that can inhibit the formation of the RNA-protein complex (Rouault, Hentze et al. 1989). Moreover, as tagging of RNA is only applicable for *in vitro* studies, the complexes formed may not reflect those which could have built under native conditions.

The incorporation of a well-characterised protein binding sequence into RNA during *in vitro* or *in vivo* transcription, which is applied for the system which is based on the interaction the MS2 coat protein and its cognate RNA (Bardwell and Wickens 1990), may result in inefficient elution of the purified complex, as this specific RNA-protein interaction is supposed to be very stringent (Das, Zhou et al. 2000). Alternatively, a protease cleavage site might be introduced to directly release the protein of interest from the column upon proteolytic processing (Leonov, Sergiev et al. 2003). Tagging of a specific RNA may also

be achieved by hybridisation with affinity-tagged biotinylated oligonucleotides. These are complementary to accessible single-stranded regions of the target RNA but have limited application in highly structured RNAs (Blencowe, Sproat et al. 1989; Walker, Scott et al. 2008).

In summary, all of the methods described, imply their very own disadvantages. However, the StreptoTag method for *in vivo* applications, as established here, may nevertheless provide added value. First, the Strepto-tags allow synthesis of even highly structured RNAs *in vivo*. This is particularly important as regulatory proteins often require formation of complex secondary structures to allow binding (Magin-Lachmann, Hahn et al. 2001). Second, as no further modifications have to be made, the RNA-protein complex as established under physiological conditions can be readily loaded onto the affinity column and analysed under mostly native conditions. Finally, considering its limitations, the StreptoTag method should now facilitate to shed light on novel RNA-protein interactions as they occur *in vivo*.

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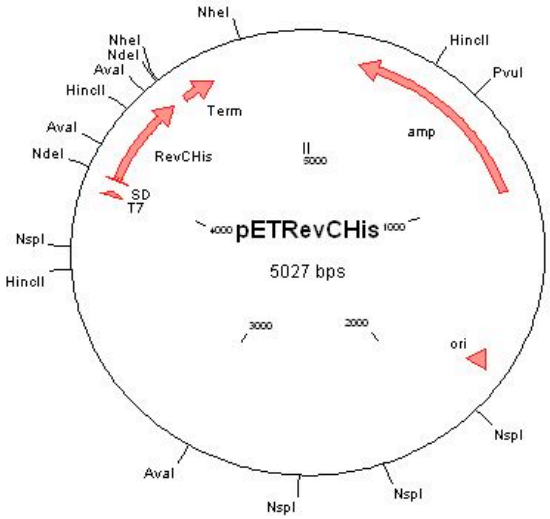
8. Appendix

8.1 Abbreviations

x	times
x g	times gravitational acceleration
°C	degree Celsius
A	adenine
aa	amino acid
BSA	bovines serum albumin
bp	base pair
C	cytosine
cm^2	square centimetre
DEPC	diethylpyrocarbonate
dH_2O	distilled water
ddH_2O	double distilled water
DMEM	Dulbeccos's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotid triphosphate
DTT	dithiothreitol
EDTA	ethylene-diamine- tetraacetate
env	envelope
G	guanine
gag	group specific antigen
HIV	human immunodeficiency virus
hr	hour
HRP	horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	kilo bases
kV	kilo volts
L	litre
LD	loading dye
LTR	long terminal repeat
M	molar
mA	milliamp
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MMTV	Mouse mammary tumor virus
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
Nef	negative factor
NES	nuclear export signal
ng	nanogram
NLS	nuclear localisation signal
nm	nanometre
nM	nanomolar
nt	nucleotide
OD	optical density
O/N	over night
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonylfluoride
pol	polymerase
PSB	protein sample buffer
Rem	regulator of export of MMTV mRNA
Rev	regulator of the expression of the virion
RNA	ribonucleic acid
rNTP	ribonucleotid triphosphate
rRNA	robosomal ribonucleic acid
RRE	Rev responsive element
RmRE	Rem responsive element
rt	room temperature
sec	second
Stag	StreptoTag
TEMED	tetramethylethylenediamine
Temp	temperature
tRNA	transfer ribonucleic acid
U	unit
UTP	uridine triphosphate
UV	ultra violet
V	volt
v/v	volume to volume ratio
v/w	volume to weight ratio
wt	wild type
µg	microgram
µl	micro litre

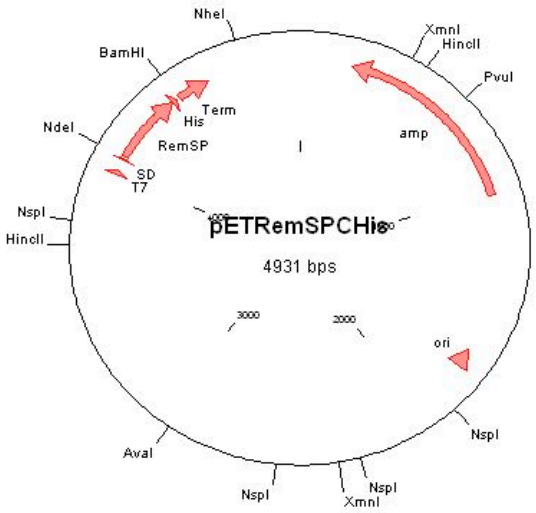
8.2 Plasmids

pETRevCHis : IPTG-inducible procaryotic expression vector encoding C-terminally histidine-tagged HIV-1 Rev protein.



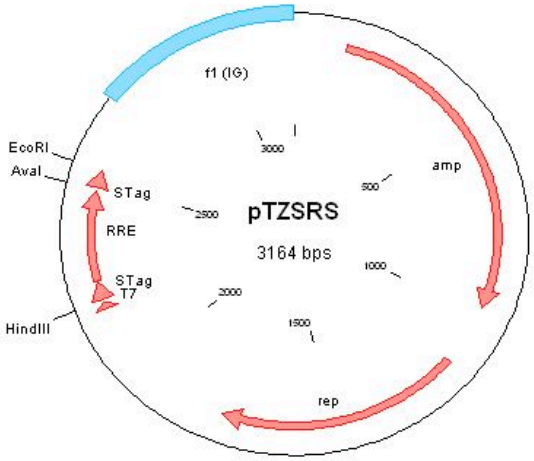
T7 : Promoter recognized by T7 polymerase; **SD** : Shine-Dalgarno-sequence recognized by procaryotic ribosomes and start of translation; **sRevCHis** : C-terminal histidine-tagged HIV-1 Rev protein; **Term** : Termination signal recognized by bacterial ribosomes and end of translation; **amp** : β-lactamase encoding ampicillin resistance gene; **ori** : origin of replication

pETRemSPCHis : IPTG-inducible procaryotic expression vector encoding C-terminally histidine-tagged MMTV Rem protein.



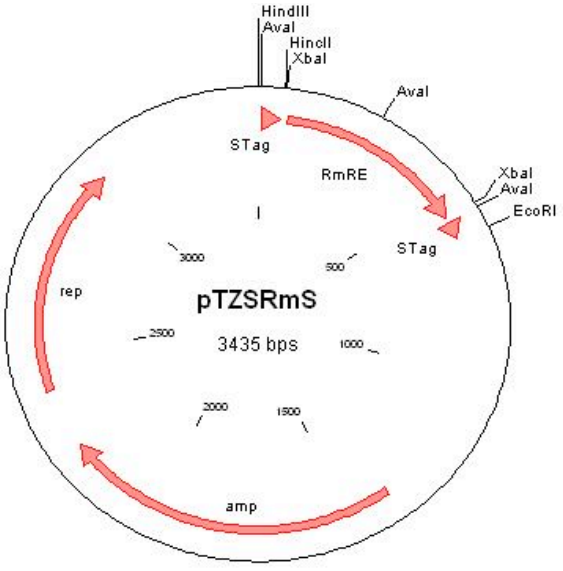
T7 : Promoter recognized by T7 polymerase; **SD** : Shine – Dalgarno – Sequence recognized by procaryotic ribosomes and start of translation; **RemSP** : 14 kDa cleavage product of full – length MMTV Rem protein; **Term** : Termination signal recognized by bacterial ribosomes and end of translation; **amp** : β-lactamase encoding ampicillin resistance gene; **ori** : origin of replication

pTZSRS : Vector for *in vitro* transcription of double Strepto-tagged HIV-1 RRE-RNA.



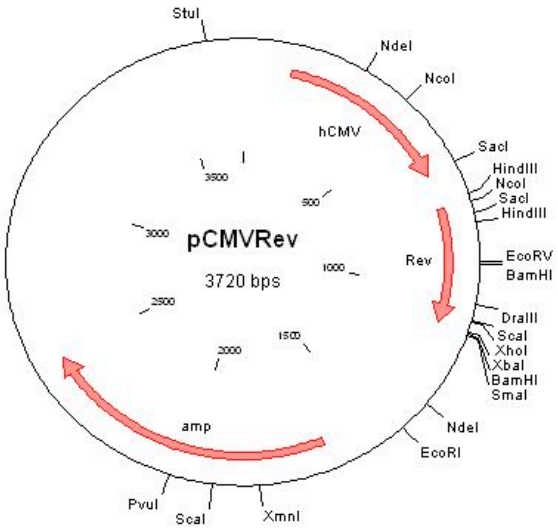
T7 : Promoter recognized by T7 polymerase; **STag** : StreptoTag; **RRE** : HIV-1 Rev responsive element; **f1 (IG)** : origin of replication of bacteriophage f1; **amp** : β-lactamase encoding ampicillin resistance gene; **rep** : origin of replication

pTZSRmS : Vector for *in vitro* transcription of double Strepto-tagged MMTV RmRE-RNA.



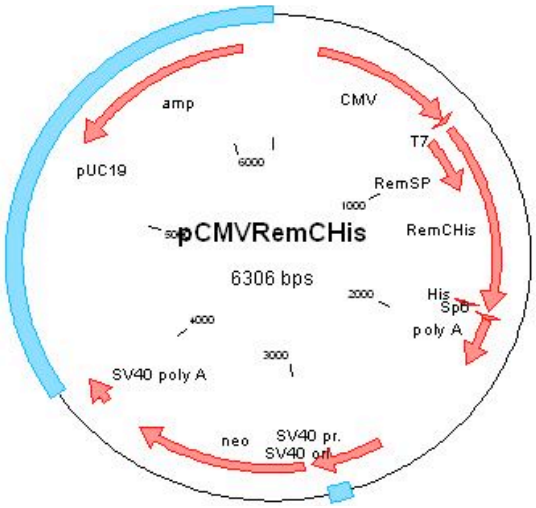
STagT : StreptoTag; **RmRE** : MMTV Rem responsive element; **amp** : β-lactamase encoding ampicillin resistance gene; **rep** : origin of replication

pCMVRev : Eucaryotic expression vector encoding HIV-1 Rev protein.



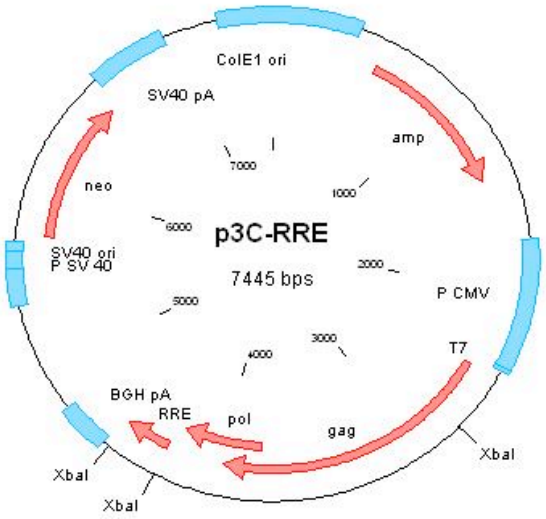
hCMV : promoter of human Cytomegalovirus; **sRev** : HIV-1 Rev protein; **amp** : β-lactamase encoding ampicillin resistance gene;

pCMVRemCHis : Eucaryotic expression vector encoding C-terminally histidine-tagged MMTV Rem protein.



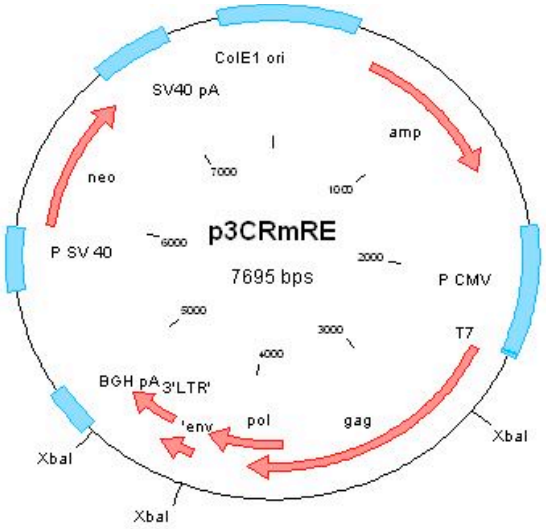
CMV : promoter of human Cytomegalovirus; **MCS** : Multiple cloning site; **T7** : Promoter recognized by T7 polymerase; **SP** : 14 kDa cleavage product of full – length MMTV Rem protein; **RemCHis** : C-terminally histidine-tagged MMTV Rem protein; **poly A** : polyadenylation signal **SV40 pr.** : SV40 early promoter; **SV40 ori** : SV40 origin of replication; **SV40 poly A** : SV40 polyadenylation signal; **amp** : β-lactamase encoding ampicillin resistance gene; **neo** : Neomycin resistance gene

p3C-RRE : Eucaryotic expression vector encoding HIV-1 Rev responsive element RNA.



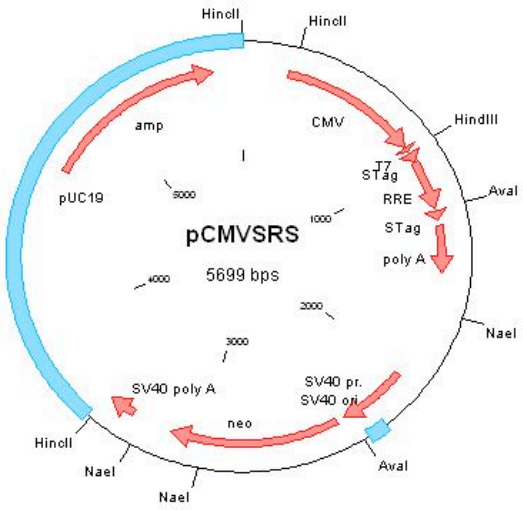
P CMV : promoter of human Cytomegalovirus; **T7** : Promoter recognized by T7 polymerase; **gag** : group - specific antigen; **pol** : polymerase; **RRE** : Rev responsive element; **BGH pA** : bovine growth hormone polyadenylation signal; **P SV40** : SV40 early promoter; **SV40 ori** : SV40 origin of replication; **SV40 poly A** : SV40 polyadenylation signal; **amp** : β-lactamase encoding ampicillin resistance gene; **neo** : Neomycin resistance gene; **ColE1 ori** : origin of replication

p3CRmRE : Eucaryotic expression vector encoding MMTV Rem responsive element RNA.



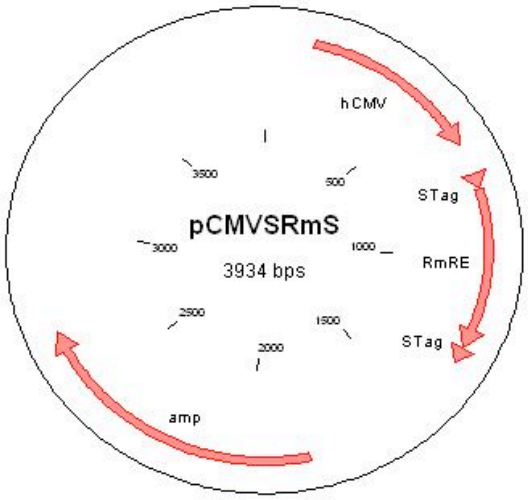
P CMV : promoter of human Cytomegalovirus; **T7** : Promoter recognized by T7 polymerase; **gag** : group - specific antigen; **pol** : polymerase; **env** : envelope; **3' LTR** : MMTV 3' Long terminal repeat; **BGH pA** : bovine growth hormone polyadenylation signal; **P SV40** : SV40 early promoter; **SV40 ori** : SV40 origin of replication; **SV40 poly A** : SV40 polyadenylation signal; **amp** : β-lactamase encoding ampicillin resistance gene; **neo** : Neomycin resistance gene; **ColE1 ori** : origin of replication

pCMVSRS : Eucaryotic expression vector encoding double Strepto-tagged HIV-1 RRE-RNA.



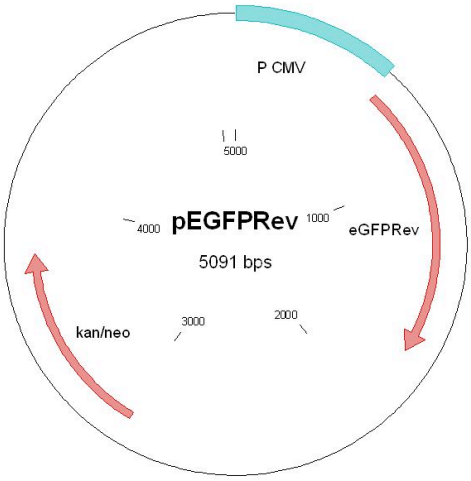
CMV : promoter of human Cytomegalovirus; **T7** : Promoter recognized by T7 polymerase; **STagT** : StreptoTag; **RRE** : HIV-1 Rev responsive element; **poly A** : BGH polyadenylation signal; **SV40 pr.** : SV40 early promoter; **SV40 ori** : SV40 origin of replication; **SV40 poly A** : SV40 polyadenylation signal; **amp** : β-lactamase encoding ampicillin resistance gene; **neo** : Neomycin resistance gene;

pCMVSRmS : Eucaryotic expression vector encoding double Strepto-tagged MMTV RmRE-RNA.



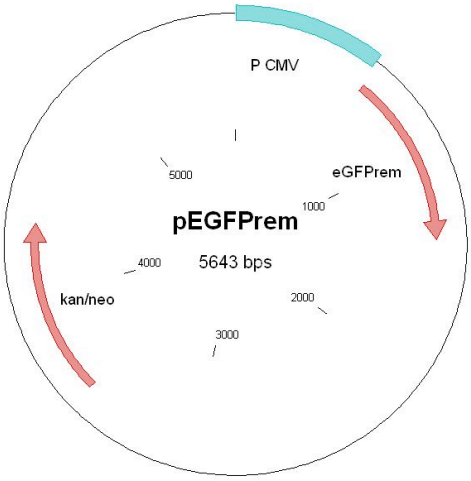
hCMV : promoter of human Cytomegalovirus; **STag** : StreptoTag; **RmRE** : MMTV Rem responsive element; **amp** : β-lactamase encoding ampicillin resistance gene;

pEGFPRev : Eucaryotic expression vector encoding EGFP-tagged HIV-1 Rev fusion protein.



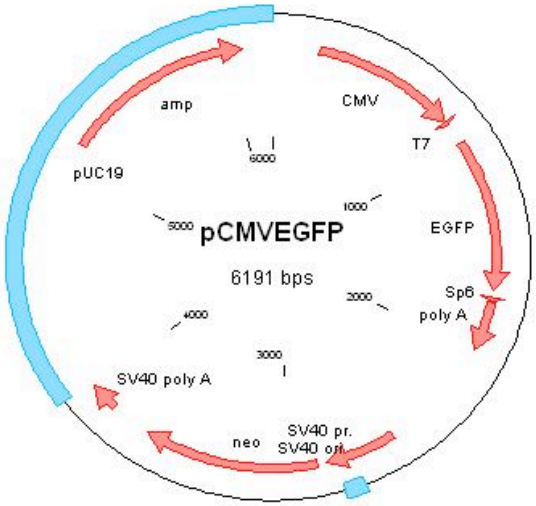
P CMV : promoter of human Cytomegalovirus; **eGFPRev** : enhanced green fluorescent protein fused to HIV-1 Rev; **kan/neo** : kanamycin and neomycin resistance gene;

pEGFPRem : Eucaryotic expression vector encoding EGFP-tagged MMTV Rem fusion protein.



P CMV : promoter of human Cytomegalovirus; **eGFPRem** : enhanced green fluorescent protein fused to MMTV Rem; **kan/neo** : kanamycin and neomycin resistance gene;

pCMVEGFP : Eucaryotic expression vector encoding enhanced green fluorescent protein EGFP.



CMV : promoter of human Cytomegalovirus; **MCS** : Multiple Cloning Site; **EGFP** : enhanced green fluorescent protein; **Sp6** : Sp6 promoter; **poly A** : polyadenylation signal; **SV40 pr.** : SV40 early promoter; **SV40 ori** : SV40 origin of replication; **SV40 poly A** : SV40 polyadenylation signal; **amp** : β-lactamase encoding ampicillin resistance gene; **Neo** : Neomycin resistance gene;

List of plasmids

name	origin
pETRevCHis	Müllner Matthias
pETRemSPCHis	Müllner Matthias
pTZSRS	Müllner Matthias
pTZSRmS	Glanz Stephan
pCMVRev	Müllner Matthias
pCMVRemCHis	Müllner Matthias
p3C-RRE	Müllner Matthias
p3C-RmRE	Müllner Mathias
pCMVSRS	Müllner Matthias
pCMVSRmS	Glanz Stephan
pEGFPRev	Indik Stanislav
pEGFPRem	Indik Starnislav
pCMVEGFP	Indik Stanislav

8.3 List of primers

PCR performed in results, 5.3.1 :

“Primer pair A” :

(forward primer)

5’ - TAT ATC TAG ACA GTG GGA ATA GGA GCT TTG - 3’

(reverse primer)

5’ - AGG AGC TGT TGT CCT TTA GGT ATC - 3’

PCR performed in results, 5.3.2 :

“Primer pair B” :

(forward primer)

5’ - CGG ATC CGG AGT GAA GGC TTT AAA TC – 3’

(reverse primer)

5’ – CAA ATG CGA TCC CTG TGG TCC TTG C – 3’

8.4 List of antibodies

antibody	species	dilution	origin
α-HIV-1 Rev	sheep	1:4500	Fitzgerald
α-HIV-1 Rev	mouse	1:500	NIBSC Unaids
α-HIV-1 Rev	mouse	1:500	NIBSC Unaids
α-HIV-1 p24	mouse	1:1000	Polymun
α-MMTV Rem	mouse	pre-diluted	Hochman J.
α-EGFP	mouse	1:1000	Clontech
α-mouse	rabbit	1:10000	DAKO
α-sheep	mouse	1:1300	DAKO

8.5 List of cell lines

HEK293 cells (ATCC CRL-1573) :
The HEK293 cells are a hypotriploid cell line originating from human embryonic kidneys, which was established by transformation with adenovirus type 5 (Louis et al., 1997). A subcultivation of 1:10 to 1:20 and a medium renewal 2-3 times per week is recommended by ATCC.

CrFK cells (ATCC CCL-94) :
This cell line is derived from feline kidneys (Crandell et al., 1973). A subcultivation of 1:3 to 1:8 and a medium renewal 2-3 times per week is recommended by ATCC.

8.6 Chemicals and Reagents

2-Mercaptoethanol	Sigma-Aldrich
Acetic acid	Merck
Acetone	Merck
Acrylamide 29:1	Bio-Rad
Agarose	Invitrogen
Agar, bacterial grade	Difco
Ammonium persulfate	AppliChem
Ampicillin	Sigma-Aldrich
Aqua bidestillata	Mayerhofer
Bovine serum albumin (BSA)	Promega
Bromphenol blue	Sigma-Aldrich
Deoxyribonucleotide triphosphates (dNTPs)	Promega
Diethyl pyrocarbonate (DEPC)	Sigma
Dimethyl sulfoxide (DMSO)	Fluka
Dithiothreitol (DTT)	Sigma-Aldrich
Dulbecco’s modified Eagle’s medium (DMEM)	Invitrogen
Ethanol	Sigma-Aldrich
Ethylene-diamine-tetraacetate (EDTA)	Sigma-Aldrich
Ethidium bromide	Sigma-Aldrich
Foetal calf serum	Invitrogen
Formaldehyde	Sigma-Aldrich

Glycerine	Sigma-Aldrich
Glycine	Sigma-Aldrich
IGEPAL	Sigma-Aldrich
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Serva
Imidazole	Merck
Isopropanol	Sigma-Aldrich
Lipofectamine 2000	Invitrogen
Magnesium chloride	Sigma-Aldrich
Methanol	Sigma-Aldrich
3-(N-morpholino)propanesulfonic acid (MOPS)	Inotech
Phenylmethanesulfonylfluoride (PMSF)	AppliChem
Phosphate buffered saline (PBS)	AppliChem
Potassium chloride	Sigma-Aldrich
Protease inhibitor cocktail	Sigma-Aldrich
RNA loading dye (2x)	Fermentas
Select peptone 140	AppliChem
Sepharose 6B	GE Healthcare
Sodium acetate	Sigma-Aldrich
Sodium chloride	Merck
Sodium dodecyl sulfate	Sigma-Aldrich
Sodium hydroxide	Merck
Sodium hydrogen carbonate	Merck
Sodium phosphate	Merck
Streptomycin	Sigma-Aldrich
Dihydrostreptomycin	Sigma-Aldrich
Orange G	Merck
Tetramethylethylenediamine (TEMED)	Bio-Rad
Tris	AppliChem
Tris-base	AppliChem
Triton X-100	Merck
0.05% Trypsin-EDTA	Invitrogen
Tween 20	Bio-Rad
Xylene cyanol	Merck
Yeast extract	AppliChem

8.7 Curriculum vitae

Personal information

Surname / First name	**Glanz Stephan**
Address	Nordmanngasse 23/1/9, 1210 Vienna , Austria
Telephone	(+43)/699 11 78 80 74
E-mail	stephan.glanz@chello.at
Nationality	Austrian
Date of birth	10 Feb.1984
Mother tongue	German
Other languages	English : excellent French : basics Italian : basics

Education

Since January 2009	Diploma thesis Institute of Virology at the Veterinary University of Vienna
Since September 2004	Magister study of Gene- and Biotechnology (equivalent Master of Science) Examination fields : molecular pathology and biotechnology. University of Vienna, Vienna Biocenter, Max F. Perutz Laboratories, IMBA

Research experience

August 2008-December 2008	Internship, Institute of Virology, establishment of a novel approach for the isolation of viral RNA binding proteins *in vivo*. Institute of Virology at the Veterinary University of Vienna

Presentations

September 2010	**Stephan Glanz** , Matthias Müllner, John Dangerfield, Walter H. Günzburg, Stanislav Indik, Christine Hohenadl. The StreptoTag method *in vivo* – proof of principle. ÖGMBT Meeting 2010, Vienna (Poster presentation)
September 2009	**Stephan Glanz** , Matthias Müllner, John Dangerfield, Walter H. Günzburg, Stanislav Indik, Christine Hohenadl. Affinity chromatography-based isolation of RNA binding proteins in physiological conditions. ÖGMBT Meeting 2009, Innsbruck (Poster presentation)
June 2009	**Stephan Glanz** , Matthias Müllner, John Dangerfield, Walter H. Günzburg , Stanislav Indik, Christine Hohenadl. The *in vivo* StreptoTag method: a new technique for isolation of RNA binding proteins. FEBS Meeting 2009, Prague (Poster presentation)

Skills and competences

- Autonomy : During my diploma work I was independently preparing progress reports and Journal Clubs. Practical obstacles were presented and discussed in Lab meetings. I took the opportunity to present my data several times at scientific meetings.

- Team work : I appreciated to work in an international and interdisciplinary team which helped me to target and overcome the many obstacles I encountered in all day research life. The input of scientists from multiple disciplines I made contact with on congresses provided me with different viewing angles helping me to overcome practical challenges.

Other work experiences

February 2006 onwards	Quality Control & Documentation Glanz Electrics
March 2005 onwards	Co-Founder of sailing club to promote offshore sailing Sailing4fun
July 2004 onwards	Skipper and organizer of offshore regattas with numerous top rankings in international events Round Palagruza Cannonbal 2007-10, BMW Hochseemeisterschaften, Jabuka Race,.
Summer 2004	Truck Catering VIA Vienna International Airport
September 2003 - May 2004	Military Service: Truck driver; Border Protection at Kittsee Austrian Military

References

1 PD Dr. Christine Hohenadl
Manager R&D Virology/Viral Vaccines II
Baxter Biosciences
e-mail : christine_hohenadl@baxter.com
Phone : +43 1-201004390 (office)
Phone : +43 6801278892 (mobile)

2 O.Univ. Prof. Dr. PhD. Walter Günzburg
Head of the Institute of Virology
University of Veterinary Medicine Vienna
e-mail : Günzburg@sgaustria.com
Phone: +65 9108 6742 (mobile)
Phone: +43 664 910 8672 (mobile)

3 Mag. Matthias Müllner
PhD student, direct supervisor
Institute of Virology,
University of Veterinary Medicine Vienna
e-mail : matthias.muellner@vetmeduni.ac.at
Phone: +43 650 368 5292 (mobile)