**BABES-BOLYAI UNIVERSITY**
**FACULTY OF BIOLOGY AND GEOLOGY**
**DOCTORAL SCHOOL OF INTEGRATIVE BIOLOGY**

Doctoral thesis

# Exploring the mobilization potential of hominoid PVA and FVA VNTR-composite elements and the cellular proteins interacting with SVA RNA in humans

Summary

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**Keywords**: retrotransposons, VNTR-composed elements, SVA, mobilization potential, cellular traffic, proteome.

# 1. INTRODUCTION

## 1.1 Transposable elements

### 1.1.1 Classification

Transposable elements are mobile repetitive DNA sequences that can move inside the genome or sometimes between genomes (Cordaux & Batzer, 2009). Two large categories of mobile elements are described based on their strategy of transposition: the DNA transposons and the retrotrotransposons (Craig, 2015). DNA transposons are mobilized by a "cut-and-paste" mechanism and they have the capacity to excise themselves from the genome and insert into a different location within the genome (Craig, 2015; Parhad & Theurkauf, 2019). The strategy used by retrotransposons is known as "copy-and-paste" because these elements are first transcribed, their RNA is reversely transcribed and inserted in a new location into the genome (Craig, 2015; Parhad & Theurkauf, 2019).

Retrotransposons can also be subdivided into two categories based on the presence or absence of long terminal repeats (LTR) in LTR- and non-LTR retrotransposons. This last class comprises the long interspersed nuclear elements (LINEs) and the variable number of tandem repeats (VNTR) composite elements (SINE-R-VNTR-*Alu* [SVAs]) (Savage et al., 2019).

Retrotransposons that are encoding for the proteins they need for mobilization are called autonomous (the LINE1 [L1] group) and retrotransposons that depend on the proteins encoded by other elements are known as non-autonomous (SINEs and SVAs depend on L1).

L1 elements are transcribed by RNA polymerase II which recognizes its promoter in the 5'UTR. These elements contain two open reading frames (ORF1 and ORF2). ORF1 encodes a RNA binding protein while ORF2 encodes a 150 kDa protein with both reverse transcriptase and endonuclease activity required for L1 mobilization (Lavasanifar et al., 2019; Moran & Gilbert, 2002).

SVA elements are composite retrotransposons flanked by target site duplications (TSDs) and contain a CCCTCT hexameric simple repeat region [(CT)n] at their 5' end, followed by the *Alu*-like region (composed of two antisense *Alu* fragments homologous to *Alu* elements), the VNTR domain (of 35–50 bp) and a SINE-R (SINE of retroviral origin) element (Wang et al., 2005).

### 1.1.2 Mobilization of SVA elements

SVA elements are mobilized *in trans* by the L1 protein machinery (Raiz et al., 2012) and are likely to be transcribed by RNA polymerase II (Wang et al., 2005). The mobilization efficiency of SVA elements was shown to be significantly attenuated when the (CT)n hexamer simple repeat and the *Alu*-like region were deleted suggesting an important role of this region in retrotransposition (Raiz et al., 2012; Hancks et al., 2012).

### 1.1.3 Interaction of L1 elements with cellular factors

The cellular factors that are interacting with L1 RNA in the cell are from different functional categories like RNP complex, RNA splicing, transcription and post-transcriptional regulation, ubl conjugation, host virus interaction or mRNA stability were found (Goodier, et al., 2013).

The retrotransposition of L1 elements was observed to be inhibited by many proteins like the APOBEC3 proteins (Wissing et al., 2011; Liang et al., 2016), MOV10 helicase (Li et al., 2013), the SAMHD1 enzyme (Hu et al., 2015), the zinc-finger antiviral protein (Moldovan & Moran, 2015), PABPN1 and PABPC1 (Dai et al., 2012). SAMHD1 was on was also demonstrated to have an inhibitory effect on the retrotransposition of SVA elements (Zhao et al., 2013).

## 1.2 PCBP1 and PCBP2 proteins

The poly(C)-binding proteins (PCBPs) are nucleic acid binding proteins interacting with poly(C) DNA and RNA which are implicated in many biological processes like mRNA stabilization, translational silencing and translational enhancement (see reviews of Choi et al., 2009; Geuens, Bouhy, & Timmerman, 2016; Makeyev & Liebhaber, 2002). PCBP1 and PCBP2 proteins contain three domains (hnRNP K-homology [KH]) responsible for the nucleic acid binding, two of them are localized at the N terminus of the protein, followed by an intervening sequence of variable length and the third KH domain at the C terminus (Du et al., 2005).

PCBP2 was previously reported to be part of the L1 ORF1 protein complexes and was identified in both nuclear and cytoplasmic extracts (Goodier et al., 2013). This protein was shown to be co-localized with ORF1 protein and LARP1 in cytoplasmic granules of 2102Ep cells.

## 1.3 The TREX complex

The proteins of TREX complex firstly identified in yeast where they regulate the transcription of mRNA, its processing, decay, and nuclear export, are highly conserved throughout metazoan evolution (for review see Katahira, 2012). This multi-protein complex

(termed THO) is composed of Thoc (Thoc1, Thoc2, Thoc3, Thoc5, Thoc6, and Thoc7) and UAP56, ALY/REF and CIP29 proteins (for review - Delaleau & Borden, 2015). Regarding a role of the TREX complex in the traffic of mobile elements in the cell, it was shown in *Drosophila* germline, that Thoc5 and other TREX components are important in the biogenesis of small non-coding RNAs that control the expression of transposable elements (Hur et al., 2016). The TREX complex is not required for yeast Ty1 retrotransposition, moreover, its presence may inhibit the insertion of these elements into the genome (Manhas et al., 2018).

# AIMS OF THE THESIS

The aims of this study were: i) to investigate the mobilization potential of two new non-autonomous retrotransposons (PVA and FVA composite elements) that were identified in the genome of the gibbon and ii) to explore the composition of SVA RNA-associated proteome and its possible roles in the turnover of these mobile genetic elements.

# 2. MATERIALS AND METHODS

## 2.1 Bacterial culture, transformation and plasmid isolation

### 2.1.1 Bacterial strains

The bacterial strain used for cloning procedures in this study was *Escherichia coli* strain DH5α (Grant, Jesseet, Bloomt, & Hanahan, 1990).

### 2.1.2 Culture conditions of *E. coli*

Bacteria were cultivated overnight in LB medium at 37°C and 200 RPM or on LB-agar plates at the same temperature without mixing. LB-agar was mixed with ampicillin (Roth, Karlsruhe, Germany), which was the antibiotic used for selection of transformed bacteria, at a final concentration of 50 mg/l.

### 2.1.3 Preparation of competent bacteria, transformation and selection

Competent cells were prepared using the protocol published by Chung and Miller (Chung & Miller, 1993).

Bacterial cells were transformed by a chemical method. All the vectors used contain the ampicillin resistance gene which is used as a selection marker in bacterial cells.

## 2.2 Plasmid DNA isolation

### 2.2.1 Minipreps

For analytical purposes that did not require high amounts of DNA (e.g. control digestions or sequencing) the plasmid DNA purification was performed based on selective alkaline denaturation of chromosomal DNA, while low molecular weight circular DNA remains double stranded (Birnboim & Doly, 1979). This method uses sodium dodecyl sulfate (SDS) and sodium hydroxide for lysis and denaturation, followed by neutralization with potassium acetate which renaturates DNA, cause the formation of aggregates from DNA, proteins and RNA, and allows us to purify plasmid DNA which remains in the supernatant.

### 2.2.2 Midipreps

For human cells transfection, *in vitro* transcription and further cloning for which a higher concentration of plasmid DNA was needed, isolation was performed using peqGOLD XChange Plasmid Midi kit (Peqlab, Erlangen, Germany). This midiprep kit uses anion exchange columns from which the DNA is eluted and then purified by isopropanol precipitation, followed by ethanol wash.

## 2.3 Culture of eukaryotic cells and retrotransposition assay

### 2.3.1 Cell line and culture condition

For the *in vivo* experiments, HeLa HA cells were used that were kindly provided by J. Moran (University of Michigan Medical School, Michigan, USA).

HeLa HA cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland) 4.5 g/l Glucose supplemented with 10% FCS (Lonza, Basel, Switzerland and Biowest, Nuaille, France), 2 mM glutamine (Lonza, Basel, Switzerland) and 100 U/ml Pen-Strep (Lonza, Basel, Switzerland).

### 2.3.2 Cell-based retrotransposition assay

The mobilization potential of domain swaps was tested by using a cell-based retrotransposition assay based on the use of a *mneoI* reporter cassette (Freeman et al., 1994; Moran et al., 1996). The retrotransposition potential was tested in HeLa HA cells, using as a driver human L1RP (Kimberland et al., 1999). The structure of the reporter cassette assures that the transfected cells will be resistant to neomycin only when the domain swaps are transcribed, spliced and re-integrated into the genome.

## 2.4 Nucleic acid analysis

### 2.4.1 Reverse transcription

Synthesis of cDNA was performed on total RNA isolated from HeLa cells, using either SuperScript II reverse transcriptase (Cat. No. 18064, Invitrogen, Carlsbad, USA) or Verso cDNA kit (Cat. No. AB1453A, Thermo Scientific, Waltham, USA).

### 2.4.2 *In vitro* transcription

To obtain the target RNA that should be used for RNA pull-down assay, *in vitro* transcription was performed. First, vectors that contain the target sequence were linearized with a restriction enzyme (single cutter) and next the *in vitro* transcription reaction was prepared, according to the specifications of the producer. The riboprobes used for Northen blotting labelled with biotin were generated by *in vitro* transcription, adding biotin-16-UTP along with ATP, CTP, and GTP.

### 2.4.3 RNA Immunoprecipitation (RIP)

RIP is based on the precipitation of a specific RNA binding protein together with its RNA partner. This technique was used in this study to confirm the binding of PCBP1 and PCBP2 to SVA RNA.

First, HeLa HA cells were seeded on 150 mm Petri dish. Cells were transfected 24 hours later either with the expression vector that contains SVA element (pcDNA3SVA) or with the vector without SVA (pcDNA3) for control. The next day, RIP was performed following the next steps: preparation of magnetic beads for immunoprecipitation, the lysate preparation and the RIP assay.

## 2.5 Northern blotting

Northern blotting was performed using the NorthernMax-Gly kit from Life Technologies (Cat. No. AM1946, Carlsbad, USA) following the main steps described by the manufactures. The main advantage of this kit is that it uses glyoxal/dimethylsulfoxide (DMSO) to denature the RNA; therefore, the use of formaldehyde was avoided.

## 2.6 SVA RNA half-life evaluation

The half-life of SVA RNA was evaluated using actinomycin D. First, HeLa cells were seeded on T25 flasks and were transfected with PCBP2 siRNA or scrambled siRNA and after 24 hours the cells were transfected with pCEP4 SVApA. Following another 24 hours from this second transfection, the cells were split in six 3.5 cm plates in which the next day was added

actinomycin D at a final concentration of 1µg/ml and total RNA was isolated at the following time points: 0h, 2h, 4h, 6h, 8h and 10h.

### 2.7 Protein isolation

To obtain cell lysates, different protocols were used based on the following applications that were needed. For RNA pull down assay, the protein extraction protocol used was published (Leppek & Stoecklin, 2014) and it mainly assumes the isolation of proteins from 20 x 150 mm Petri dishes with confluent HeLa Ha cells by using liquid nitrogen and 5 mm steel balls for 5 in a cryomill (Retsch, Haan, Germany).

### 2.8 Molecular cloning

#### 2.8.1 Plasmid constructs

To determine the structural part of VNTR composite elements that is responsible for mobilization, we constructed domain swaps between SVA (pADSVA_E [Raiz et al. 2012]), PVA and FVA. For creation of domain swaps, we used the $(CT)_n$ hexametric repeats and *Alu*-like domain from one element and we combined it with the VNTR and 3'part of another element.

### 2.9 Transfections

#### 2.9.1 siRNA transfection

To determine the importance of the identified proteins in the mobilization process, the mobilization rate was assessed after the expression of these proteins was inhibited by siRNA. This double stranded RNA is approximate 20–25 bp in length and interferes with the mRNA of specific genes leading to degradation and no translation in proteins. The gene silencing was measured 48 and 72 hours after the transfection.

#### 2.9.2 Plasmid DNA transfection

For the cell-based retrotransposition assay for assessment of mobilization in *trans* we used the protocol previously described by (Raiz et al. 2012)

After transfection, the cells were incubated for 24h, and then the medium was changed to medium with hygromycin. The hygromycin selection continued for 12 days, and then the cells were trypsinized and transferred in medium with G418, followed by staining with Giemsa after 12 days.

### 2.10 Analysis of RNA-protein interactions

#### 2.10.1 RNA pull-down assay

To identify proteins that interact with the RNA of SVA elements we choose a system that uses four copies of a S1 modified aptamer (S1m), that needs to be cloned 3' of the target sequence. The method that uses this system is called RNA pull-down assay and was used based on a protocol published by Leppek (Leppek and Stoecklin 2014). RNA pull-down assay assumes binding of the RNA of interest to agarose or sepharose beads, followed by binding of proteins to the already bound RNA and finally elution of the bound proteins with RNase.

The proteins we obtained from the pull-down assays were first visualized on polyacrylamide gels (SDS-PAGE) and then were prepared for identification through mass spectrometry.

## 3. RESULTS

### 3.1 The structure of the CT-*Alu*-like region determines retrotransposition potential

To test if the specific sequence and structure of the 5' domain (CT repeats and *Alu*-like) of VNTR elements is responsible for retrotransposition, we created domain swaps by fusing the CT/*Alu*-like domain from the SVA elements with the VNTR and 3'part of the PVA and FVA elements (SP and SF) and the CT/*Alu*-like domain from the PVA and SVA elements was fused to the VNTR and 3'part of the SVA element (PS and FS). The retrotransposition rate of SP and SF elements was higher compared to PVA and FVA and the PS and FS elements had a reduced mobilization activity compared to the SVA elements.

### 3.2 Splicing as the mechanism of assembly of PVA and SVA elements

As splicing was identified as the possible mechanism responsible for the formation of PVA and FVA elements (Ianc et al., 2014) we wanted to see if there may be additional splicing processes that could have an impact on the total quantity of full-length *mneoI*-spliced RNA and thus explaining the low mobilization potentials for the two elements. Northern blotting analysis helped us identify a second donor splice site within the VNTR domain and an acceptor splice site at the 3′-end of the neomycin phosphotransferase (*neo*) ORF (Ianc et al., 2014) further supporting the computational prediction results obtained (Ianc et al, 2014). A large amount of full length *mneoI*-spliced RNA available for retrotransposition for the PVA and FVA elements was observed, so their low mobilization rate cannot be assigned to the RNA availability. However, a smaller amount of full length *mneoI*-spliced RNA was observed for the FS element, so in this case there may be a link between the RNA availability and the mobilization potential.

# 4. DISCUSSIONS

## 4.1 PVA and FVA elements

In the genome of the gibbon, four families of VNTR composed elements flanked by TSDs were identified ( Carbone et al., 2012; Carbone et al., 2014; Ianc et al., 2014). All four famillies of VNTR elements have the same structure represented by CCCTCT hexameric repeats, followed by an *Alu*-like domain, the VNTR region and a 3' part which is variable between families (Ianc et al., 2014). PVA elements contain at their 3' end a unique sequence characteristic for SVA2 elements, followed by a fragment from the prostaglandin reductase 2 gene (*PTGR2*) (exon 4 and the 5' part of intron 4) (Ianc et al., 2014). FVA elements are characterized by a 3'end that contains a part of a FRAM (Free Right *Alu* Monomer) element flanked by non-repetitive sequences (Ianc et al., 2014).

## 4.2 Mobilization of FVA and PVA elements

PVA and FVA elements identified in the *N. leucogenys* genome, were seen at a low copy number, of 143 and 11, respectively (Ianc et al., 2014), suggesting that there might be a specific region within these elements which inhibits their mobilization or they are interacting with some inhibitory factors. The hexameric (CCCTCT)n repeats and *Alu*-like region of SVA elements were previously shown to be crucial in retrotransposition (Raiz et al., 2012; Hancks et al., 2012).

## 4.3 Structural determinants of the retrotransposition of PVA and FVA elements

Domain swaps were created between SVA and PVA/FVA by exchanging the (CCCTCT)n repeats and *Alu*-like regions. The chimeras with the hexamer CT repeats and *Alu*-like region from the SVA and VNTR+3' region from the PVA and FVA elements are efficiently mobilized, similar to SVA elements, as compared to chimeras with 5' part from the PVA and FVA and the VNTR+3'region from SVA elements which have a mobilization rate similar to full length PVA and FVA elements. Based on these results and on specific deletions observed in the *Alu*-like region of LAVA and SVA elements which are still active (Ianc et al., 2014), we can conclude that not the presence of the hexamer CT repeats and *Alu*-like region is important in efficient mobilization of composite elements, but their specific structure.

# CONCLUSIONS, NOVELTY OF RESEARCH AND PERSPECTIVES

This study confirms the significance of the 5' part (CT/*Alu*-like domain) in retrotransposition of these non-autonomous mobile genetic elements.

Until now, the host factors interacting with the SVA elements in the cell were not characterized. Here, we provide a first study describing proteins that are interacting with SVA

RNA. This study can be considered a starting point for future confirmation of the other proteins we identified and their impact on retrotransposition.

To gather more information on the traffic of VNTR composed elements in the cell, would require to conduct the same experiments as described here, but using *in vitro* transcribed PVA and FVA RNA instead of SVA.

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