Introducing The CAZL Element: A Minimal 3' UTR Sequence That Imparts Enhanced Expression To Transgenes In Target Cells

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What is the CAZL element?

CARe = Cytoplasmic accumulation region

ZCCHC14 binding loop (ZL)

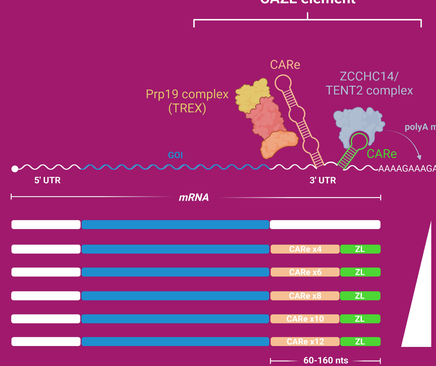
10nt repeat:

CCAGAUCCUG



Synthetic consensus predicted to bind TREX complex proteins for nuclear export

HepBV PRE | Woodchuck HV (w)PRE | Human Cytomegalovirus RNA2.7

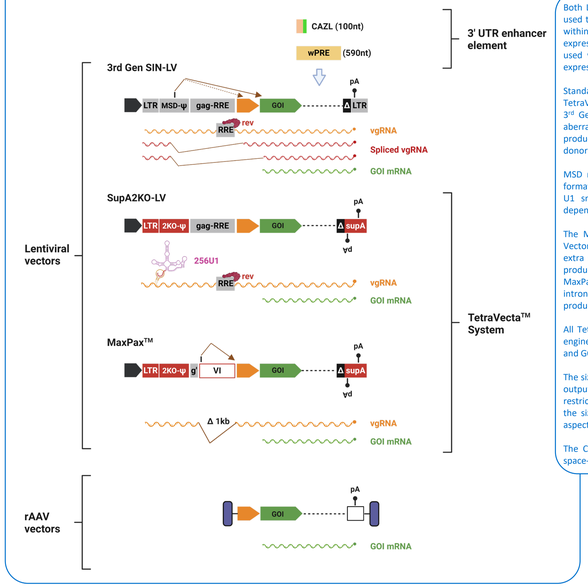


The CAZL is a composite of a nuclear export element[1-2] and a ZCCHC14 binding loop that mediates poly-A mixed tailing to reduce mRNA turnover[3].

References
1. Lei et al. 2011 DOI: 10.1093/nar/gks1314
2. Lei et al. 2013DOI: 10.1073/pnas.1113076108
3. Kim et al. 2020DOI: 10.1038/s41594-020-0427-3

Some images created with BioRender.com

① Viral vector genomes used to exemplify CAZL



Both Lentiviral (LV) and rAAV vector genomes were used to assess the effect of the composite elements within the CAZL element on gene of interest (GOI) expression. Typically, the wPRE 3' UTR element is used within viral vector cassettes to enhance GOI expression in target cells.

Standard 3rd Generation and next generation TetraVecta™ System LV genomes were used. Unlike 3rd Gen LVs, TetraVecta genomes do not generate aberrantly spliced vector genomic (vg)RNA during production, due to inactivated of the major splice donor (MSD).

MSD mutation leads to reduced full length vgRNA formation but this is rescued by the expression of a U1 snRNA-based molecule (256U1) for RRE/Rev-dependent LVs.

The MaxPax™ LV genome replaces the RRE for a Vector-Intron (VI). This means it has additional ~1kb extra space for transgene sequences, and can be produced independently of Rev and 256U1. MaxPax™ LVs cannot carry transgene cassette introns, due to their splicing-out during vgRNA production.

All TetraVecta LV genomes also harbour supA-LTRs, engineered for improved transcriptional insulation and GOI expression from integrated cassettes.

The size of viral vector genome is a vital factor for LV output titres, and critically for rAAVs, which is strictly restricted to ~5kb. Therefore, the ability to reduce the size of all *cis*-acting sequences is an important aspect of vector design.

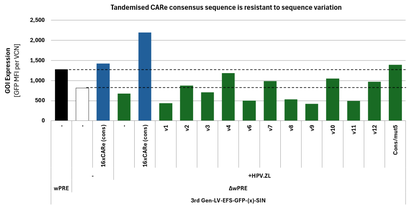
The CAZL is a 3' UTR GOI enhancer element with space-saving as a key metric.

② CAZL characterisation: CCAGATCCTG repeat is key

Consensus mutants



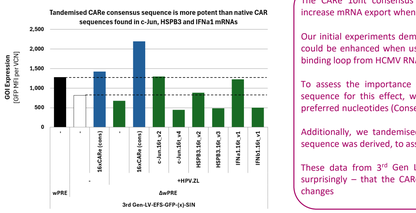
CCAGTTCCTG	Consensus CARe
TCAGTTCCTG	Variant 1
GCAGTTCCTG	Variant 2
CAAGTTCCTG	Variant 3
CCTGTTCCTG	Variant 4
CCTGCTCCTG	Variant 6
CCTGTACCTG	Variant 7
CCTGTTGCTG	Variant 8
CCTGTTCGTG	Variant 9
CCTGTTCCAG	Variant 10
CCTGTTCCTC	Variant 11
CCAATTCCTG	Variant 12
CCAGTTCCTG-CCAGTTCCga	Cons/mut5



Native variants



CCAGTTCCTG	Consensus CARe
CCTGAACCTG	c-Jun v2
CAAGAACGTG	c-Jun v4
CCAGTTCCTC	HSPB3 v2
CCAGATGGTG	HSPB3 v3
CCAGTTCCAG	IFNa1 v1
GAAGCTCCTG	IFNb1 v1



The CARe 10nt consensus sequence was previously shown by others to increase mRNA export when tandemised within 5' UTRs (refs 1-2).

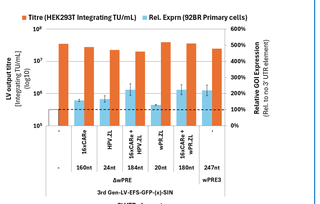
Our initial experiments demonstrated that GOI expression from LV cassettes could be enhanced when using a 16x CARe sequence fused to the ZCCHC14 binding loop from HCMV RNA2.7 (HPV.ZL; ref 3) in the 3' UTR.

To assess the importance of the nucleotides within the CARe consensus sequence for this effect, we systematically changed each position for less preferred nucleotides (Consensus mutants).

Additionally, we tandemised native CAR sequences, from which the CARe sequence was derived, to assess their relative functionality within the CAZL.

These data from 3rd Gen LV-transduced Jurkat cells indicate – somewhat surprisingly – that the CARe consensus is relatively intolerant to sequence changes

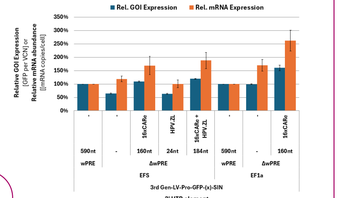
③ ZLs from HPV and wPRE are comparable



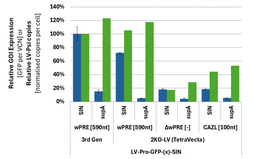
Next we compared the minimal ZLs from HCMV RNA2.7 and wPRE in the context of the 16x CARe, producing 3rd Gen LVs harbouring an EFS-GFP cassette. Output titres were broadly similar, indicating that RRE/rev export of vgRNA dominates over the TREX-mediate nuclear export pathway used by CAZLs.

Transduction of primary cells at matched MOI demonstrated that CAZLs based on either ZLs were equally active, and equivalent to the shortened wPRE3 element, despite the CAZL's shorter length.

④ CAZL increases GOI mRNA copies per cell



⑤ CAZL pairs with supA-LTR



In follow-on experiments in 3rd Gen LV-transduced Jurkat cells at matched MOIs, we analysed the relative abundance of GOI mRNA copies per cell, and normalised to vector copy-number.

The presence of the CAZL was associated with an increase in GOI mRNA copies per cell, for both EF1a (+intron) and EFS (no intron) promoter driven cassettes.

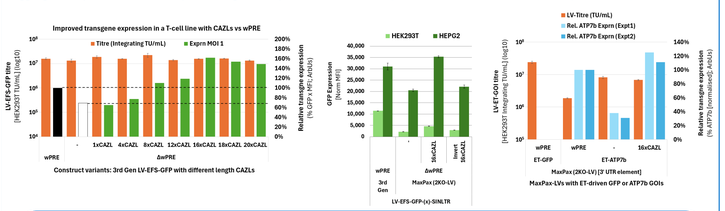
Separately, we assessed the impact of the CAZL within 3rd Gen and 2KO-LVs containing either standard SIN-LTRs or the improved supA-LTRs. HEK293T cells were transduced at matched MOIs and both GFP expression and 5' transcription read-in measured and normalised to vector copy-number.

The CAZL element did not increase LV backbone transcription, and imparted a modest increase in GOI expression when paired with supA-LTRs.

⑥ CAZL imparts equivalent/better GOI expression than wPRE in T-cell and Liver cell lines

Toggling GOI expression in Jurkats by increasing CARe repeats

Combining MaxPax™ and CAZL for therapeutic gene delivery in HepG2 cells



We generated a number of 3rd Gen LVs encoding EFS-driven GFP with 3' UTR CAZL variants, where the number of CARe 10nt consensus repeats were increased from just 1x to 20x. These LVs were produced at equivalent titres as measured by integration assay, and then used to transduce Jurkat cells at an MOI=1. GFP Expression scores were generated by flow cytometry and normalised to vector copy-number per cell.

These were plotted relative to a wPRE-containing vector (black bar). The CAZLs imparted increased GFP expression as CARe repeat number increased, and this plateaued at the 16xCAZL, which had 160% expression compared to wPRE.

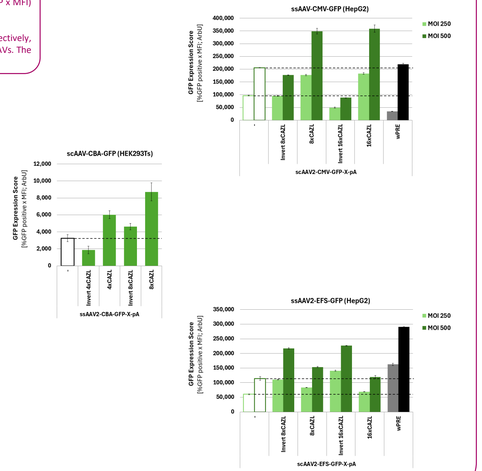
The 16xCAZL was cloned into the MaxPax™ (TetraVecta) LV encoding EFS-GFP or ATP7b (copper-transporting ATPase 2 for Wilson's Disease) driven by the liver-specific ET promoter. CAZLs mediated their greatest effect in HepG2 cells, and in an orientation-specific manner. CAZLs appear to be less functional in HEK293T cells for LV-delivered cassettes. MaxPax-ET-ATP7b titres were also greatest for cassettes encoding the 3' CAZL compared to the larger wPRE element, perhaps reflecting the smaller vgRNA size packaged.

The 4x, 8x and 16x CAZL elements were cloned into self-complementary (sc)AAV2 genome plasmids or single stranded (ss)AAV2 genome plasmids, and rAAVs produced in HEK293 cells by triple transfection (i.e. with pRepCap2 and pHelper).

Crude rAAV samples were titrated by qPCR before transducing target cells at matched MOIs. ssAAV2-CBA-GFP vectors were used to transduce HEK293T cells. The ssAAV2-CMV/EFS-GFP vectors were used to transduce HepG2 cells. GFP Expression scores (%GFP x MFI) were generated 3 days after transduction.

The 4xCAZL and 8xCAZL scAAV2-CBA-GFP vectors enabled 2-fold and 3-fold greater GFP expression in HEK293Ts respectively, compared to no 3' UTR element, or inverted controls. Similar results were observed in HepG2 cells using 8x/16x CAZLs by ssAAVs. The wPRE element had a greater impact on EFS-driven GFP compared to CMV, whereas CAZLs were universally impactful.

⑦ CAZLs increase GOI expression from rAAVs



Summary

- There is a strong desire to minimise *cis*-acting sequences within viral vector genomes without loss of function.
- We have developed 100-200nt 3' UTR transgene enhancer elements called 'CAZLs'. These are based on a tandemised 10nt cytoplasmic accumulation region (CARe) and the ZCCHC14 binding loops from post-transcriptional elements (PRE) such as from wPRE.
- CAZLs are shorter in length compared to the full length wPRE (590nt), but in certain target cells such as T-cell and Liver cell lines can enhance GOI expression to similar or greater levels compared to wPRE.
- CAZLs increase the level of GOI mRNA, and can be tailored to suit a specific expression level in target cells.
- CAZLs are shorter novel 3' UTR enhancers for rAAVs and for LVs with larger payloads where every transgene nucleotide counts!

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