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Screening and structural engineering of lariat-capping ribozymes for use as an alternative mRNA 5`-capping system

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Background. RNA is becoming an important therapeutic modality, as witnessed with ascendance of mRNA vaccines. Despite some successes, the mRNA production, especially in scale, is still challenging. Large-scale production of translationally-competent therapeutic mRNAs requires efficient 5'-capping, which is realized by either co-transcriptional capping with cap analog nucleotides, or by additional post-transcriptional enzymatic reaction, both of which either introduce considerable increase in production costs or production process complexity. Promising alternative strategy is to use lariat capping ribozymes (LCR) to shield 5'-end of RNAs from nucleases combined with internal ribosome entry site (IRES) to engage protein expressing machinery. **Aim.** Identification of native and structurally-engineered variants of LCRs with faster processing kinetics and increased lariat capping, and their use for mRNA translation in cells. **Methods.** We tested native and mutated LCRs by *in vitro* processing kinetics assay and native MS-based RNAseH cleavage-assisted capping efficacy assay. The lead variants were subcloned into constructs with either CVB3 IRES or EMCV IRES, coupled to Gaussia luciferase as a reporter and polyA motif, transcribed and electroporated into HepG2 cells. The relative reporter accumulation in culture media 72h post transfection was assayed by luciferase glow assay. **Results.** Two sets of LCR variants were tested: orthologs derived from various protists, assembled by OE-PCR with oligonucleotides (from Yuria-Pharm), and set of sequence variants (generated by SDM) of LCR from *Didymium iridis* (from GenScript), designed to modify thermodynamic stability of P2 stem or invade P10 stem in active confirmation. The variants with higher processing rates (3-fold increase) and higher capping efficacy (increase from 45% to 93%) were identified, which confirmed the key role of fast lariat product release from post-transesterification complex for an increase in the ratio of capped to cleaved species. The LCR variants with highest reaction rates and capping efficacy were further tested in capless translation system described before and demonstrated up to 4-fold increase in the accumulation of luciferase in comparison to IRES-only configuration, while still being 6-fold less active than ARCA-capped transcripts. In context of different IRES, both overall effect size and ranking of LCR variants were significantly different, highlighting mutual dependence on the sequence/folding context. **Conclusions.** The capless translation system constructed from functionally-coupled lariat capping ribozyme and viral IRES significantly increased overall protein production compared to IRES-only configuration, while still inferior to co-translational capping with ARCA. Structural engineering of DiLCR stems by modulation of their thermodynamic stability, allowed us to control lariat cap/cleavage products ratio and design variants with near-quantitative capping achievable *in vitro*, leading to increased protein accumulation in the cell-based translation assay. Alternative LCR-IRES reporter combinations demonstrate high dependence of functional activity on sequence context possibly due to the mutual folding/interaction environment interference.

Keywords: cap analogs, mRNA production, lariat-capping ribozyme