Synthesis of 1-Methyl-7-Nitroisatoic Anhydride and Analysis of *gurken* mRNA by Selective 2'- Hydroxyl Acylation Analyzed by Primer Extension Chemistry

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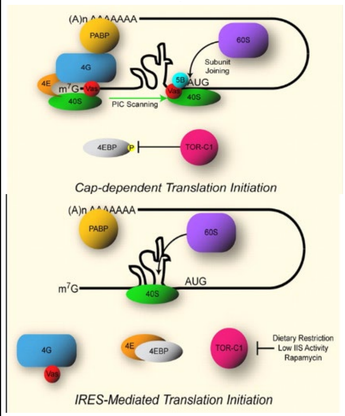
Abstract

We are analyzing *gurken* mRNA from *Drosophila melanogaster* through Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension (SHAPE) chemistry to evaluate secondary structure of an Internal Ribosomal Entry Site (IRES). RNA is linearized and folded, before being introduced to one of three electrophillic reagents. The reagents (1-methyl-7-nitroisatoic anhydride (1M7), N-methylisatoic anhydride (NMIA), and 1-methyl-6-nitroisatoic anhydride (1M6)) detect local nucleotide flexibility by reacting with 2'- hydroxyl groups. At conformationally flexible positions, the RNA is reactive, but where nucleotide base pairing has occurred, the 2' hydroxyl region is unreactive. Reverse transcription with a fluorescently labeled primer produces populations of cDNA fragments at reagents adduction points. Capillary electrophoresis is used to measure local flexibility by reading terminated cDNA fragment populations, and the data determines the secondary structure through QuSHAPE and RNA structure software. We will describe the synthesis and the spectroscopic characterization of 1M7 which we used to distinguish complex secondary structures in the RNA.

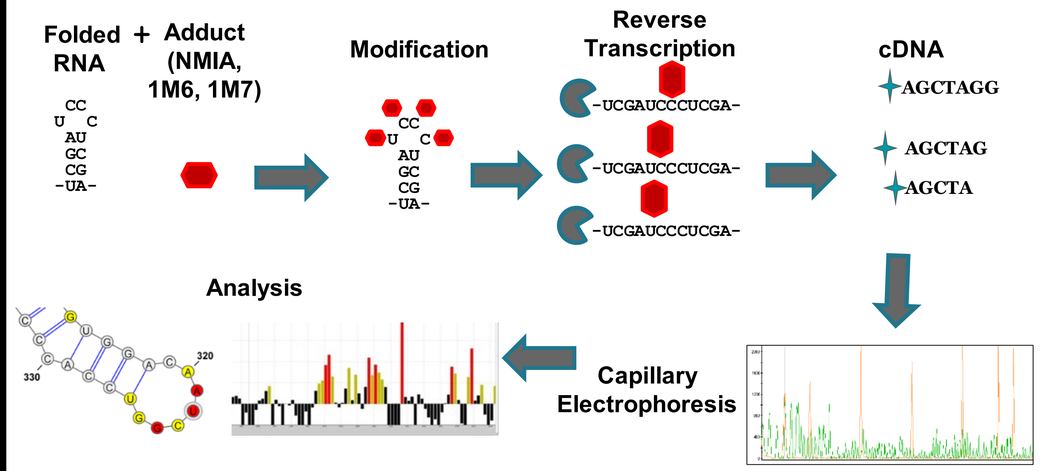
Introduction: *Gurken* mRNA IRES

Internal Ribosomal Entry Sites have highly specific sequences and secondary structures allowing for ribosomal subunit recruitment and cap-independent translation. *Grk* is a TGF-α related signal ligand in *Drosophila*, which binds to the Epidermal Growth Factor Receptor (EGFR). Its expression is vital for proper axis specification in an oocyte.[3] When nutrient availability is sufficient, *gurken* can be translated using the cap-dependent pathway. However, an IRES will function when nutritional sources are not abundant in the mother's environment or the flies are fed Rapamycin. The IRES becomes activated as there is a high concentration of unused ribosomes surrounding the mRNA.[4] Using SHAPE chemistry on the *gurken* 5' UTR, the secondary structure of the the IRES can be identified. Differnetial SHAPE can be performed with three reagents, 1-methyl-7-nitroisatoic anhydride (1M7), N-methylisatoic anhydride (NMIA), and 1-methyl-6-nitroisatoic anhydride (1M6). 1M7 reacts rapidly with the RNA and is useful in detecting complex secondary structures such as pseudoknots.

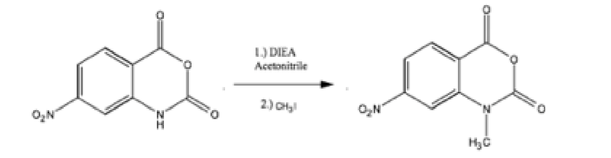


Translation Initiation of *gurken* mRNA. (Top) Typical cap-dependant translation initiated by the m^7G cap and numerous trans-acting initiation factors. These factors together recruit the 40S ribosomal subunit to begin translation. (Bottom) Nutrient scarcity or Rapamycin treatment initiate the use of and IRES due to the blocking of cap-dependant translation. Excess ribosomes around the IRES allows for direct recruitment of the 40S to begin cap-independent translation. [4]

Methods: Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension[2]

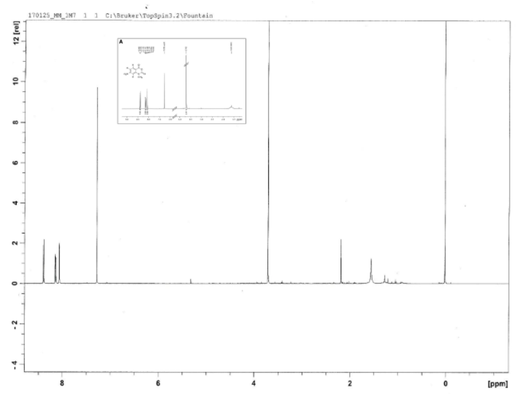


Synthesis of 1-methyl-7-nitroisatoic anhydride[1]



Reaction Scheme For 1M7 synthesis. In the first step, 4-nitroisotic anhydride (4NIA) is reacted with DIEA to abstract a proton from the nitrogen in the 4NIA ring. Next, the nucleophilic nitrogen becomes methylated by attacking the methyl iodine, where iodine acts as a leaving group.

NMR Characterization of 1-methyl-7-nitroisatoic anhydride



Spectroscopic Characterization of 1M7(Left). Nuclear Magnetic Resonance analysis was performed on the product to confirm the identity of 1M7. Proton NMR of 1M7 in chloroform: 3.703 (s, 3H), 8.062 (d), 8.145 (dd, 1H), 8.396 (d, 1H). The peak at 7.27ppm is an internal solvent standard. (Inset) Accepted NMR spectroscopy for 1M7. [1]

1M7 Synthesized Samples (Right). A 1M7 unreacted sample has a yellow coloration. (Left Tube) and a 1M7 reacted sample has a red coloration (Right Tube).

Electropherogram

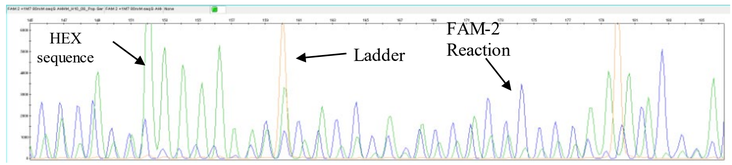


Figure 3: Electropherogram of 1M7. Following the SHAPE reaction of FAM-2 and 1M7, this electropherogram was generated with a 3500 Genetic Analyzer. The green colored peaks represent the sequencing that was used, which was HEX. The orange colored peaks are the ladder that we used. The blue represents the actual reaction of the FAM-2 in combination with 1M7.

Current Structure[2]

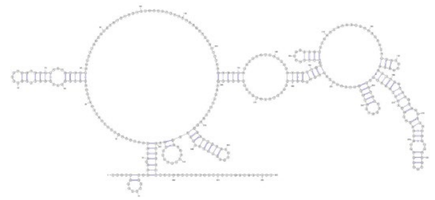


Figure 1: gurken IRES secondary structure. Based on all of the runs that have been completed, this is the current secondary structure of the gurken IRES. Raw data was gathered off of the analyzer and was run through a program called QuSHAPE. This program analyzes the raw data to get the SHAPE score. Following that, we used a program called RNAstructure to create the hypothetical structure based on the SHAPE data.

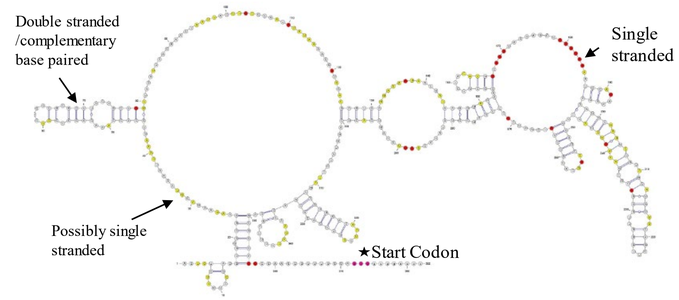


Figure 2: 1M7 interactions. Using our current structure, we reacted the gurken RNA with 1M7. The variety of colors represent different interaction that the 1M7 had with the structure. Red bases indicate highly reactive nucleotides, yellow indicates moderate reactivity. The uncolored nucleotides represent unreactive bases. The start codon is labelled in pink.

References

1. Turner, R., Shefer, K., and Manuel, A. (2017). Safer One-Pot Synthesis of the 'SHAPE' Reagent 1-methyl-7-nitroisatoic anhydride (IM7). *RNA Journal*, 1857-1860.
2. Martin, A., Emborski, C. (2017) Unpublished Data. State University of New York at Fredonia, Fredonia, NY.
3. Thio, G., R. Ray, G. Barcelo and T. Schüpbach (2000). Localization of gurken in Drosophila oogenesis requires elements in the 5' and 3' region of the transcript. Developmental Biology 221: 435-446.
4. Ferguson, S.B., Blundon, Malachi A., Klobstand, Martha S. and Schüpbach, Trudi (2012). Modulation of gruken translation by insulin and TOR signaling in Drosophila. State University of New York at Fredonia, Fredonia, NY.