SilentMutations (SIM): a tool for analyzing long-range RNA-RNA interactions in viral genomes and structural RNAs

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ABSTRACT

Background: A single nucleotide change or mutation in the coding region can alter the amino acid sequence of a protein. In consequence, natural or artificial sequence changes in viral RNAs may have various effects not only on protein stability, function and structure but also on viral replication. In the last decade, several tools have been developed to predict the effect of mutations in structural RNA genomes. Some tools employ multiple point mutations and are also taking coding regions into account. However, none of these tools was designed to specifically simulate the effect of mutations on viral long-range interactions.

Results: Here, we developed SilentMutations (SIM), an easy-to-use tool to analyze the effect of multiple point mutations on the secondary structures of two interacting viral RNAs. The tool can simulate destructive and compensatory mutants of two interacting single-stranded RNAs. This will facilitate a fast and accurate assessment of key regions, possibly involved in functional long-range RNA-RNA interactions and finally help virologists to design appropriate experiments. SIM only needs two interacting single-stranded RNA regions as input. The output is a plain text file containing the most promising mutants and a graphical representation of all interactions.

Conclusion: We applied our tool on two experimentally validated influenza A virus and hepatitis C virus interactions and we were able to predict potential double mutants for in vitro validation experiments.

Availability: The source code and documentation of SIM are freely available at github.com/desiro/silentMutations

Key words: Codon Mutation, Virology, RNA virus, Virus Bioinformatics, RNA secondary structure, double-mutant, silent mutation

Introduction

In the last decades, several computational tools for the analysis of RNA secondary structures have been developed. However, tools specifically targeting virus needs are still rare and underresearched1-3. For example, long-range RNA-RNA interactions (LRIs) play an important role in the life cycle of RNA viruses. Many LRIs are already known to directly functioning as activators or inhibitors of viral replication and translation4. For instance, several interactions were recently identified as possibly new LRIs in the hepatitis C virus (HCV) genome using LRIscan5. Some of these computationally identified interactions have already been verified experimentally6-13. Apart from long-range interactions forming in a single sequence, LRI-like structures can also occur between two separate sequences. Whereas the general function of such interactions is still under investigation, they can be seen as viral LRIs and are presumably responsible for the correct packaging of all segments into the viral capsid of segmented viruses such as the influenza A virus (IAV)14-17. Due to the rapidly growing number of possible functional LRIs identified, it is essential to have an effective verification method. Technically, such interactions can be destroyed by mutation or removal of the interacting parts using in vitro experiments8,18. Such sequence changes can result in secondary structure changes and manifest different viral titers. However, with the alteration of the sequence, unwanted effects can arise that not only effect the LRI. To cope with this issue, we can alter both interacting RNA parts simultaneously. Finally, we want
the combination of both mutated parts resulting in a similar interaction strength in comparison to the interaction between the wild type (WT) sequences. A single mutated sequence part combined with the opposing WT sequence should destroy the interaction. Such a technique was recently used to verify a possible LRI between two IAV segments. Several computational tools are available that can alter a sequence and report alternative secondary structures with one or even multiple mutations. However, these tools are designed for certain applications and do not meet the requirements of the previously proposed experimental technique: the combinatorial in vitro analysis of RNA-RNA interactions.

In this study, we present a tool called SilentMutations (SIM) that effectively simulates synonymous (silent) compensatory mutations in two single-stranded viral RNAs and is therefore appropriate for the in vitro assessment of predicted LRIs.

Materials and Methods

Here, we present a command-line tool, called SilentMutations (SIM), that can simulate synonymous structure-destroying and structure-preserving mutation pairs within coding regions for long-range RNA-RNA interaction experiments. The tool has been written in Python (v3.6.5) and relies heavily on the RNAcofold python site-package of the ViennaRNA Package (v2.4). As the ViennaRNA Package is available for Linux, Windows, and MacOS, SIM runs on all three different platforms. The various parameters of SIM are fully adjustable and several filters (Fig. 1) allow a fast and accurate prediction, therefore the tool can be run on a standard notebook. A simple run takes about 20 seconds when using the standard parameters, two sequences of length 21 as input, and a single core. Generally, the runtime highly depends on the parameter setup and the length of the input sequences. The main computational steps of SIM are (1) preprocessing, (2) permutation, (3) attenuation, (4) recovery, and (5) sampling.

A special challenge in respect to negative single-stranded viruses (such as Ebola, Marburg, and Coronaviruses) is, that most databases only contain the positive strand of the viral genomes. While the protein-coding sequence is encoded on the positive strand, the folding of these viruses happens on the negative strand. Depending on the user input, SIM can automatically create the reverse and complement strand of negative single-stranded RNA viruses for folding while maintaining codon integrity on the positive strand. This can be easily achieved with the --virusclass=ssRNA-, --reverse, and --complement options. The user can directly provide the positive strand sequence from the database in a fasta file, with the reading frame and regions on the positive strand. The default output of SilentMutations prints all relevant information of the folding in a plain text file and additionally creates for each folding a VARNA command for visualization. Additionally, if the tool can find installations of VARNA and inkscape, it will directly generate high-quality vector graphics of the various foldings. A default binary of VARNA (v3.93) is provided with the tools source code. SilentMutations is primary designed for two sequence snippets (hereafter called snips) in coding regions but can be used to simulate interactions between coding and non-coding regions as well as for interactions between two non-coding regions. This can be exercised with the --noncoding1 and --noncoding2 parameters, denoting the first or second sequence as non-coding, respectively.

We use the following notation for the minimum free energy (mfe) obtained by folding sequence y with sequence x via RNAcofold:

$$mfe(x, y)$$

The mfe is represented as a negative value in kcal/mol and therefore having a lower mfe results in a more stable structure.

Preprocessing

The first step of SIM extracts sequence snips from a longer sequence template while keeping codons intact (Fig. 1). Especially, the difficult extraction of snips in the exact reading frame from negative sense single-stranded RNAs can be easily handled by the build-in functionalities of the tool. SIM thus directly accepts the whole sequence as input, together with a start and end position for the snips and the reading frame. The tool first extracts the requested interacting parts from each sequence and automatically increases them in both directions if the provided range would otherwise split a codon based on the predefined reading frame (Fig. 1). This ensures a preservation of all codons. Both sequences, pre-snip1 (ps1) and pre-snip2 (ps2), will then be fold together via RNAcofold to acquire only codons involved in the folding. Unpaired codons at the termini will be removed to get the final snip1 (s1) and snip2 (s2) from which SilentMutations calculates the two mutant sequences mut1 (m1) and mut2 (m2).
Different sets and their abbreviations are given in brackets. Details can be found in the Methods.

Figure 1: Overall workflow of the SilentMutations tool, exemplary shown for two sequences from a negative single-stranded RNA virus genome (ssRNA-). (a) The first step will extract the defined range in each sequence and possibly increase the range to preserve codons in the given reading frame. Both so-called pre-snips are folded with RNAcofold to remove unpaired codons at the endings. We refer to an extracted single-stranded RNA snippet as snip. (b) All possible codon permutations (here, reverse complements due to ssRNA-) are generated for both snips (called perms) to conserve the amino acid sequence. Permutations with too many mutations defined by the mutations (-mut) parameter are discarded. (c) To further decrease the total number of combinations, SIM will fold each snip with all permutations of the other snip and keep permutations with a mfe higher or equal to mfe(snip1,snip2) times the filter percentage (-prc) parameter, denoted as upper limit l. (d) All remaining snip1 permutations will then be folded against all snip2 permutations to find snip1 and snip2 mutations with a similar fold mfe. A double-mutant mfe(mut1,mut2) fold is considered to be similar, if its mfe is lower than the wild-type mfe(snip1,snip2) times the lower deviation (-udv) parameter and higher than mfe(snip1,snip2) times the upper deviation (-udv) parameter. (e) The last step will minimize a combined single-mutant mfe(snip1,mut1) and mfe(snip1,snip2) by keeping both mutations in a similar range depending on the mutation range (-mrg) parameter. The range for eachmutation combination is defined by (mfe(snip1,mut1) + mfe(mut1,snip2)) · 0.5 · (1 - mrg) for the lower threshold (l-mrg) and (mfe(snip1,mut1) + mfe(mut1,snip2)) · 0.5 · (1 - mrg) for the upper threshold (u-mrg). Different sets and their abbreviations are given in brackets. Details can be found in the Methods.
Permutation

In this step all possible permutations pre-perms1 (P*) and pre-perms2 (Q*) of synonymous codons are created from each sequence in order to find the most suitable mutations that maximize the distance between mfe(s1,s2) and mfe(s1,m2) as well as the distance between mfe(s1,s2) and mfe(m1,s2), while keeping the wild-type mfe(s1,s2) and the double-mutant mfe(m1,m2) similar. The number of these permutations can be vast, depending on the number of synonymous codons and the length of the sequence. In order to reduce the computational complexity, this step also removes every permutation sequence with more mutations than predefined by the --mutations parameter resulting in the sets perms1 (P) and perms2 (Q), see Fig. 1. Having fewer alterations in a sequence with a strong attenuation effect on the viral titer additionally improves the authenticity of the mutational experiment.

Attenuation

Here, a further reduction of the computational complexity is applied while already creating valid sequences with reduced mfe folding scores. Having a small difference between the wild-type mfe(s1,s2) and single-mutant mfe(s1,m2) or mfe(m1,s2) respectively would diminish the mutational effect on the interaction. It is therefore vital to increase the distance between these mfe scores. Therefore, the user can directly specify the required distance with the filter percentage (--filterperc) parameter. The defined value is then multiplied with the mfe(s1,s2) value to obtain a upper limit (denoted l) for mfe(s1,m2) and mfe(m1,s2). Then, the tool creates all foldings between s1 and Q as well as P and s2. Each folding mfe(s1,qj ∈ Q) and mfe(pi ∈ P, s2) (with i and j defining the i-th and j-th element of set P and Q, respectively) is only considered for the next recovery step if the mfe is higher than l. The new sets mut1 (U) and mut2 (V) have a drastically reduced size compared to P and Q which greatly reduces the computational complexity for finding the two mutant sequences m1 and m2 where mfe(m1,m2) is similar to the wild-type mfe(s1,s2). While it seems to be convenient to set a very low --filterperc parameter, this could also result in an empty permutation set. On the other hand, setting a high --filterperc could result in longer running times. It is therefore recommended to start with the default 0.5 and later adjust this parameter depending on the performance.

Recovery

To find double-mutants (mut) with a similar folding mfe such as the wild-type (WT) folding mfe, each sequence ui ∈ U and vi ∈ V has to be folded against each other with RNAfold. The runtime of this folding process is highly dependent upon the previous attenuation step. When using the unprocessed P* and Q* sets from the example in Fig. 1, the number of required folding operations would be around 24 million and reduced to only 37,000 for the filtered sets U and V. Some LRIs function as inhibitors for viral replication and a stronger interaction would decrease functional viral reproduction. It is therefore necessary for the simulation of the double-mutant interaction to set a lower but also an upper similarity threshold. Each of the calculated mfe(ui ∈ U, vi ∈ V) values are therefore compared with the wild-type mfe(s1,s2). A combination of two sequences u and v is only valid if

\[ mfe(s_1, s_2) \cdot l_{dv} \geq mfe(u, v) \geq mfe(s_1, s_2) \cdot u_{dv} \]

with l_{dv} as the lower deviation (----lowerdev) and u_{dv} as the upper deviation (----upperdev) parameter. Valid (u, v) double-mutant pairs are stored as tuples in set M.

Sampling

All pairs in this final set M now only contain mutated sequence pairs with the desired properties. However, greedily selecting the i-th tuple (u, v), ∈ M that minimizes

\[ mfe(s_1, v_i \in M) + mfe(u_i \in M, s_2) \]

could result in unfavorable interaction strengths between WT's and mutants. An unfavorable v and u pair would result in a significantly higher single-mutant mfe of the mutant folded with the WT sequence in comparison to the mfe of the other mutant and WT combination. Therefore, it is recommended to keep both, mfe(s1,v_i ∈ M) and mfe(u_i ∈ M, s2), in a similar range while minimizing the combined mfe. Accordingly, the sampling step takes the mutation range (--mrg) parameter into account and calculates the lower threshold 1-mrg as

\[ (mfe(s_1, v_i \in M) + mfe(v_i \in M, s_2)) \cdot 0.5 \cdot (1 + mrg) \]

and the upper threshold u-mrg as

\[ (mfe(s_1, v_i \in M) + mfe(v_i \in M, s_2)) \cdot 0.5 \cdot (1 - mrg) \]
for each mutant tuple. The most suitable mutant pair, holding all predefined requirements, is finally found by calculating:

\[
\min\{mfe(s_1, v_i \in M) + mfe(u_i \in M, s_2)\}
\]

with

\[
1-mrg \geq mfe(s_1, v_i \in M) \geq u-mrg
\]

\[
1-mrg \geq mfe(u_i \in M, s_2) \geq u-mrg
\]

Results and Discussion

SilentMutations is primarily designed as a supporting tool in the assembly step of an in vitro experiment, such as previously performed by Gavazzi et al. in influenza A viruses\(^{15}\). With the help of SIM, experimentalists can simulate for the first time specific RNA-RNA interactions of viral coding- and non-coding sequence snippets, potentially forming stable long-range interactions. Using SIM, the search space of possible double-mutant sequences, forming a stable secondary structure with a comparable mfe to the wild-type structure and simultaneously disrupting the single-mutants structure, can be drastically reduced. Therefore, SIM can be used prior time- and cost-consuming wet-lab experiments to simulate promising double-mutant sequences that preserve the wild-type LRI, however are not functional as single-mutants. To validate SIM and to show that the tool is able to predict biological relevant mutants, we used two validated interaction examples, one in the influenza A virus H5N2 A/finch/England/2051/1991 strain and another in the hepatitis C virus type 1b strain (accession: AJ238799.1).

Application to influenza A virus

The influenza A virus (IAV) genome consists of 8 viral ribonucleoproteins (vRNPs) and each vRNP segment includes one of 8 different negative sense and single-stranded viral RNAs. It is hypothesized, that these segments are packed selectively through RNA-RNA interactions between the 8 segments\(^{16,30–32}\). To this end, Gavazzi et al.\(^{15}\) performed an in vitro mutation experiment in IAV, perfectly shaped to validate our tool. They took a yet unconfirmed interaction from one of their previous experiments\(^{14}\) between the PB1 and the NS segment and introduced four trans-complementary point substitutions by hand. An interaction between the PB1 mutant and NS mutant resulted in a similar viral titer than using the PB1 WT and NS WT segment. Introducing only the PB1 mutant or NS mutant resulted in both cases in an attenuation of the viral replication.

To show that we are able to obtain the same experimental results of Gavazzi et al.\(^{15}\), computationally, we adjusted some key parameters of SIM. Importantly, we limited the number of possible mutation pairs to four and calculated the mfe between the WT and mutant (mut) combinations in mere seconds. The SIM results of the secondary structure predictions and mfe values for the two IVA segment combinations are shown in Fig. 2. By only allowing a maximum of four mutation pairs, we were able to calculate the same structures and mutations as previously proposed by Gavazzi et al.\(^{15}\). Moreover, our results show a mfe difference of 1.80 kcal/mol between wild-type and double-mutant (Fig. 2 a and b) and a mfe difference of 2.20 kcal/mol between single-mutants (Fig. 2 c and d). Again, we want to point out that for the in silico experiment it is preferable to have a similar mfe between the two wild-type IAV segments mfe(NS\(_{WT}\), PB1\(_{WT}\)) and the double-mutant mfe(NS\(_{mut}\), PB1\(_{mut}\)) as well as between the single-mutants mfe(NS\(_{WT}\), PB1\(_{mut}\)) and mfe(NS\(_{mut}\), PB1\(_{WT}\)).

In a next step and by using SIM with default parameters, we were able to calculate a double-mutant that not only reflects the results of Gavazzi et al.\(^{15}\), but also shows slightly lower mfe differences between the wild-type and the double-mutant (1.20 kcal/mol) as well as between the two single-mutants (1.20 kcal/mol), see Fig. 3.

Therefore, the interaction strengths of mfe(NS\(_{WT}\), PB1\(_{WT}\)) and mfe(NS\(_{mut}\), PB1\(_{mut}\)) as well as mfe(NS\(_{WT}\), PB1\(_{mut}\)) and mfe(NS\(_{mut}\), PB1\(_{WT}\)) are more closely to each other in comparison to the results of Gavazzi et al.\(^{15}\). As a possible drawback, our simulation needs to introduce one more point mutation in each single-stranded RNA snip (Fig. 3).

Application to hepatitis C virus

The hepatitis C virus (HCV) genome consists of a positive single-stranded RNA of about 10 kb length. This RNA is translated into a single polyprotein that is later cleaved into four structural (C, E1, E2, p7) and six nonstructural (NS2, NS3, NS4A, NS4B, NS5A, RdRp) proteins by viral and host proteases\(^{33,34}\). Both UTR regions of the viral genome are highly structured and have been extensively studied in the past\(^{33,35–38}\).

For our validation of SIM, we have chosen a well studied interaction\(^{8}\) between the 3’ UTR and the end region of the ORF encoding the HCV
**Figure 2:** IAV interactions like previously shown by Gavazzi et al. We adjusted the parameters of SIM and allowed only four mutation pairs in maximum to calculate the mfe values for mutated sequence combinations of the NS and PB1 segments like previously shown by Gavazzi et al. The mfe values and secondary structures are shown for (a) the wild-type interaction, (b) the double-mutant interaction, (c) the first single-mutant (PB1 mut) interaction, and (d) the second single-mutant (NS mut) interaction. This visualization was automatically generated by SIM using VARNA.

**Figure 3:** IAV interactions calculated by SIM. By applying SIM to the same IAV sequence snips of the NS and PB1 segments, we were able to show lower mfe differences between (a) the wild-type interaction and (b) the double-mutant interaction, as well as between the two single-mutant interactions (c,d).

**Figure 4:** HCV interaction calculated with SIM. We used SIM to calculate a double-mutant interaction between a HCV protein-coding snip of 5BSL3.2 and an long-range interacting non-coding snip of the 3’ SLII region. Again, our calculations show similar mfe values between the stable wild-type and double-mutant structures (a,b) as well as between the structure-destroying and less stable single-mutants (c,d).
polyprotein. Several studies\textsuperscript{9,33,39–42} have already shown that the RNA replication highly depends on the conserved structures of the X-tail\textsuperscript{33,43} sequence contained in the 3' UTR. This sequence is presumed to contain three experimentally verified stem-loops (SLI, SLII, SLIII)\textsuperscript{44} which may interact with other parts of the viral genome through long-range RNA-RNA interactions to regulate replication\textsuperscript{40}. The LRI between the free nucleotides of the hairpin from the 3' SLII structure and the free nucleotides of the hairpin from the 5BSL3.2 structure have been subject of many HCV studies.

The interaction was first verified by Friebe \textit{et al.}\textsuperscript{5} and was also computationally found with LRIscan by Fricke \textit{et al.}\textsuperscript{5}. Another reason for selecting this interaction as an example was the duality of having one interacting part in a coding and one in a non-coding region. Applying SIM on this interaction resulted in two compensatory point mutations in each snip (Fig. 4). As the 3' SLII region is non-coding, only the point mutations in the snip from the 5BSL3.2 structure are silent. Our results show, that the wild-type structure mfe(5BSL3.2\textsubscript{WT}, SLII\textsubscript{WT}) should have a similar strength compared to the calculated double-mutant structure mfe(5BSL3.2\textsubscript{mut}, SLII\textsubscript{mut}), see Fig. 4. Additionally, both mfe(5BSL3.2\textsubscript{WT}, SLII\textsubscript{mut}) and mfe(5BSL3.2\textsubscript{mut}, SLII\textsubscript{WT}) weaken the interaction significantly.

We propose, that our simulated mutations in HCV may be used to verify the given long-range interaction. Taking all the new long-range interactions found by Fricke \textit{et al.}\textsuperscript{5} into account, we propose that our tool can be used to create mutation experiments for every predicted LRI to provide evidence for a biological function of these interactions. Such experiments would be especially interesting for IAV, where the exact packaging process of the vRNP segments is not yet fully understood.

\section*{Conclusion}

By presenting SIM, we provide an easy and fast way to analyze possible interactions between vRNPs. The tool can be used to heavily reduce the search space of possible synonymous mutation interactions between two RNAs. Another difficulty when creating silent IAV mutations lies in preserving the codons on the positive strand, while mutating the negative strand, and is also intercepted by SIM. Furthermore, our tool provides a significant speedup, not only in the verification of interactions in these two viruses, but also for many other virus families. Our simulations will help to gather a deeper understanding of the translation and replication processes in viruses and also how long-range interactions are regulating these.

A promising future approach would be the combined application of LRIscan and SilentMutations to detect currently unknown LRIs and to provide in the same step possible mutational verification experiments for each LRI.

\section*{Author Contributions}

DD performed the design, development and programming of the tool and wrote the main draft of the paper. MH, BI, and MM contributed in writing, discussions and proofreading of the final manuscript. All authors read and approved the final manuscript.

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