Secondary structure of the \textit{Irf7} 5'-UTR, analyzed using SHAPE (selective 2’-hydroxyl acylation analyzed by primer extension)

Yun-Mi Kim\textsuperscript{1}, Won-Young Choi\textsuperscript{2}, Chang-Mok Oh\textsuperscript{3}, Gyoon-Hee Han\textsuperscript{1} & Young-Joon Kim\textsuperscript{1,*}

Departments of \textsuperscript{1}Integrated OMICs for Biomedical Science, \textsuperscript{2}Biochemistry, and \textsuperscript{3}Biotechnology, Yonsei University, Seoul 120-749, Korea

\textbf{INTRODUCTION}

During innate immune responses against invading microorganisms, pattern-recognition receptors recognize conserved pathogen-associated molecular patterns and directly activate signaling cascades, thereby inducing the expression of various inflammatory mediators and type 1 interferons (IFNs) (1, 2). In a previous study, it was established that OAS1, a member of the OAS family, binds to the secondary structure of \textit{Irf7} mRNA and inhibits its translation (3). The IRF family contains the major transcription factors required for interferon responses, and these play roles in the cell cycle, apoptosis, oncogene regulation, and hematopoietic cell differentiation (4, 5). Along with IRF3, IRF7 is especially well known as a major regulator of type1 IFN production in response to viral infections (6-8). However, the molecular mechanism behind the OAS1-mediated translational regulation of \textit{Irf7} remains unknown.

Recently, many researchers have studied the secondary and tertiary structures of RNAs using various methods, such as the V1 and S1 nucleases or 1-methyl-7-nitroisatoic anhydride (1M7) (9-11). Recent studies have shown that “selective 2’-hydroxyl acylation analyzed by primer extension” (SHAPE) is a powerful method for analyzing single-stranded regions, allowing the prediction of secondary structure (11-14). The importance of RNA secondary structure in modulating the expression level of inflammatory mediators has been illustrated in several cases (15). In a previous study, our group suggested that the secondary structure of mRNA affected the interaction between OAS1 and the \textit{Irf7} 5'-UTR (3). However, the exact nature of the 5'-UTR required for OAS1 binding has not been established. Here, we investigated the single-stranded regions of the \textit{Irf7} 5'-UTR that are known to have suppressive effects on translational initiation. We identified five major single-stranded regions of the \textit{Irf7} 5'-UTR that overlap with the bulged regions, predicted using computer modeling of the secondary RNA structures. These results confirm the requirement for a dsRNA structure for the translational inhibition mediated by OAS1 and identified the cis-element involved in the OAS1-mediated translational regulation of \textit{Irf7}.

\textbf{RESULTS}

\textit{Irf7} 5'-UTR has a secondary structure

As demonstrated previously in our laboratory, OAS1 inhibits the translation of \textit{Irf7} by binding to the \textit{Irf7} 5'-UTR (3). Specifically, full suppressive activity was confirmed with the 1-215 fragment of the \textit{Irf7} 5'-UTR. We used a program to predict the structure of the \textit{Irf7} 5'-UTR and performed mutation analysis to confirm the requirement for several secondary structures in the translational regulation of \textit{Irf7}. To further confirm the validity of these secondary structures, we examined the biochemical properties of the \textit{Irf7} 5'-UTR. The \textit{Irf7} 5'-UTR was transcribed \textit{in vitro} and the mobility of the transcripts was confirmed using 2% agarose gel electrophoresis, which revealed several forms of the \textit{Irf7} transcript, migrating as rather blurry bands.

To determine whether the diffuse migrating properties of the UTR were caused by secondary structure, we denatured it at high temperature before electrophoresis. Although the tran-
Analysis of the secondary structure of Irf7
Yun-Mi Kim, et al.

Fig. 1. Irf7 5'-UTR analyzed on a denaturing polyacrylamide gel. pBluescript II SK(+)Irf7 5'-UTR1-215 was linearized using BamH1. Transcripts of the Irf7 5'-UTR were created using an in vitro transcript assay. (A) The newly generated transcripts appeared as two bands on a 2% agarose gel, even though we denatured them at high temperature, indicating the presence of some secondary structure. (B) A cDNA of the exact size of the Irf7 5'-UTR was visible on the denaturing gel (Fig. 1B); a clear and sharp 215-nucleotide (nt) band was seen. These result suggested that the transcripts of the Irf7 5'-UTR may have a secondary structure. To determine the secondary structure of the transcripts in detail, we then analyzed the Irf7 5'-UTR region with SHAPE to identify the single-stranded regions of the transcripts (14, 16-18).

Five acylation sites were identified on adenosine residues using 1M7
To explore the secondary structure of the Irf7 5'-UTR, we performed SHAPE experiments on the 1-215 fragment of the Irf7 5'-UTR using 1M7 dissolved in DMSO at pH 8.0 under denaturing conditions. 1M7 is known to modify RNA by nucleophilic attack on the 2'-hydroxyl group, causing formation of a 2'-O-adduct, especially in single-stranded regions, loops, or bulges that are conformationally unconstrained or in flexible nucleotide regions (11, 19, 20). Consequently, 1M7 caused acylation of the single-stranded regions of Irf7 5'-UTR transcripts. cDNAs were synthesized from 1M7-modified mRNA of the Irf7 5'-UTR using a [γ-32P]ATP-labeled probe. Acylation induced breaks in the transcripts and allowed the [γ-32P]ATP-labeled probe to extend only up to where the RNA was acylated. The newly synthesized cDNAs were then analyzed on a 15% urea-acrylamide gel. SHAPE analysis of the Irf7 5'-UTR led to the identification of five major bands (at nucleotide positions 70, 105, 158, 166, and 172), revealing single-stranded regions of the Irf7 5'-UTR (Fig. 2). Our experimental data revealed that single-stranded regions exist at the second and fourth stem structures and the region connecting the second and third stems of the Irf7 5'-UTR, and all five acylation sites were identified on adenosine residues. This is consistent with the observation that adenosine is the most re-
Analysis of the secondary structure of \( \text{Irf7} \)
Yun-Mi Kim, et al.

**Overall**, the secondary structure of the \( \text{Irf7} \) 5’-UTR was found to be the same as the computed predictions, suggesting that the stem-loop structure of \( \text{Irf7} \) UTR is a key cis-element for OASL1-mediated translational regulation.

**DISCUSSION**

\( \text{Irf7} \), a member of the IRF family, is known as a master regulator of type 1 IFN-dependent immune responses (5, 6, 8). A previous study in our laboratory found that \( \text{Irf7} \) is highly regulated by OASL1, at the mRNA level (3). We previously showed that \( \text{Irf7} \) translation is controlled by OASL1 through an interaction in the 5’-UTR (3). In this study, we analyzed the \( \text{Irf7} \) 5’-UTR to identify the secondary structure required for specific binding of OASL1. Single-stranded regions of the mRNA were modified by SHAPE, and the deduced structure based on the result was compared to the predicted secondary structure from five different structure prediction programs. We found that the identified single-stranded regions mostly matched the structures predicted by the five programs. However, the SHAPE analysis revealed that two regions located next to bulge areas were single-stranded. This discrepancy may reflect the physical property of the transcripts under the given reaction conditions. However, these weak interactions may also play regulatory roles in association with other regulatory factors, such as OASL1, which require a specific secondary structure. Together, these data indicate that the secondary structure of the \( \text{Irf7} \) 5’-UTR appears to assume the predicted structure and that the double-stranded re-
gions of the Irf7 5′-UTR are probably important elements for
the inhibition of OASL1-mediated Irf7 translation. However,
the exact OASL1 binding site remains to be determined. To
define the exact binding sequence of OASL1, it will be necessary
to analyze the binding of OASL1 to the Irf7 5′-UTR using an in
vitro binding assay and application of dimethyl sulfide foot-
printing (21), especially of the first and second stem loops.
These will contribute to understanding the molecular mecha-
nism underlying OASL1-mediated translational regulation.

MATERIALS AND METHODS

Plasmid Construction and template preparation
The full lengths of cDNA sequence encoding Irf7 5′-UTR 1-215 was subcloned from MetLuc2-Irf7 5′-UTR 1-215 into pbLuescript II SK (+) vector containing a T7 promoter using restric-
tion enzymes, Hind III and EcoR I. pbLuescript II SK (+) Irf7 5′-UTR 1-215 was linearized with BamHI.

In vitro transcription assay
pbLuescript II SK (+) Irf7 5′-UTR 1-215 was incubated with T7 polymerase (Enzymomics) and mixture (RNase-free water, rNTPs (Promega), 100 mM DTT (Invitrogen), recombinant RNasin (Promega), 10× T7 polymerase (Enzymomics) buffer) at 37°C for 1 h to generate mRNA. RQ1 DNase (Promega) was added to the mixture and the incubated was continued at 37°C for 30 min to remove the templates. The newly generated mRNAs were purified by phenol-chloroform precipi-
tation (RNase-free water 80 μl, phenol:chloroform:isooamyl al-
cohol 25:24:1, saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma) 100 μl, 100% ethanol (Sigma) 275 μl, 5 μM NH4Ac (Sigma) 10 μl, 75% ethanol 100 μl).

1M7 synthesis
According to the 1M7 synthesis protocol, 4-nitrosoic anhy-
dride (4NIA) was dissolved in 60 ml anhydrous dimethyl-
fluoride (DMF) in a 250-ml flask under N2. A mixture of so-
odium hydride (NaH) was made in 15 ml DMF in a separate
500 ml flask under N2 and stirred. The 4NIA solution was add-
ed to the mixture of NaH in DMF slowly and stirred. Subsequently, methyl iodide (MeI) was added and mixed well
at room temperature for 4 h. The mixture was poured into ice
cold 1 N HCl (100 ml) and filtered by vacuum filtration. The precipitate was rinsed twice with cold water, three times with ether, and dried overnight in an oven (purity 99.9%). Then, the dried 1M7 was dissolved in dimethyl sulfoxide (DMSO) at a final concentration 50 mM.

SHAPE modification
RNA (20 pmol in ½ TE buffer, 6 μl) was heated to denature it at 95°C for 2 min and quickly cooled on ice for 5 min. Then, 3 μl physiological-like folding buffer containing 333 mM HEPES (Sigma), 333 mM NaCl (Samchun), 33.3 mM MgCl2 (Sigma), pH 8.0, was added and incubated at 37°C for 20 min. Next, 50 μM 1M7 (1 μl) or DMSO (1 μl), as a control, was added and allowed to react at 37°C for 70 s and cooled on ice. In pre-
reactions, the mixture color changed from bright yel-
lo to dark yellow and the RNAs interacting with 1M7 were
degraded by hydrolysis. Modified RNAs were recovered by
ethanol precipitation with RNase-free water (90 μl), 5 M NaCl
(5 μl), and 100% ethanol (400 μl) at −80°C for 30 min, then,
washed with 75% ethanol and redissolved in 1× TE (10 μl).

End-labeling and primer extension
An oligonucleotide (synthesized by Bioneer) GGA CCC CCC
GGG CTG CAG GA was end-labeled with a [γ-32P] ATP using
T4 polynucleotide kinase (PNK) and 10× polynucleotide kin-
ase buffer at the 5′-hydroxyl terminus by incubating at 37°C
for 1 h, and was purified with phenol-chloroform at −20°C
for 30 min. Modified RNAs were heated at 65°C for 5 min to an-
neal and incubated with end-labeled probe at 42°C for 2 h.
The end-labeled probes were extended up to the modified sites.
The extension process was terminated by incubating at
70°C for 10 min.

Sequencing
pbLuescript II SK (+) Irf7 5′-UTR 1-215 was hydrolyzed and
used as a template for a sequencing ladder. The sequencing
ladder was prepared according to USB’s protocol. Briefly,
Sequenase reaction buffer and an appropriate primer (TTC
ACA AGT GTG ACC CAG GTA TTA GGG TG) were added to
the hydrolyzed DNA and the mixture was incubated at 65°C
for 2 min to denature and cooled at room temperature for
1530 min. Subsequently, dGTP, [α-32P] ATP, 0.1 M DTT, and
polymerase were added and the mixture was again incubated
at room temperature for 5 min. ddNTPs were added for the ter-
mination reaction and incubated at 37°C for 5 min. To in-
activate the extension, stop solution was added and incubated
at 75°C for 2 min. A model S2 sequencing gel electrophoresis
apparatus (Gibco BRL) was used to separate the extended
cDNA. All sequencing ladders were loaded directly from the
incubation at 75°C and the extended cDNA samples were de-
natured at 95°C for 5 min just before loading on a 15% urea-acrylamide- bisacrylamide gel. The samples were sepa-
rated at 900 V for 12 h and analyzed using the Bio imaging
analysis system (Fujifilm Life Sciences).

RNA structure prediction
The secondary structure of the mRNA was predicted using
Vienna fold software. Vienna fold predicts both lowest free-en-
ergy structures and base pair probabilities from RNA or DNA
sequences. The minimum free energy (MFE) method searches
for the most energetically stable structure and is calculated by
summing the total folding energies. To calculate each base
pair in a secondary structure, RNA sequences were subjected
to a function e, where e(i, j) is the energy of a base pair. If
both i and j are a pair, but not with each other, i pairs with
k1 and j pairs with k2, where i < k1 < k2 < j. For exam-
ple, if $E_{i+1} = E_{ij}$, $j$ is unpaired and if $E_{i} E_{j-1} = E_{ij}$, $j$ is unpaired. The end of a helix, defined by $i$ and $j$ in the algorithm, is considered for exterior loops or branches. Based on this algorithm, our experimental data derived from the SHAPE modification was processed and used to constrain the newly predicted secondary structure.

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