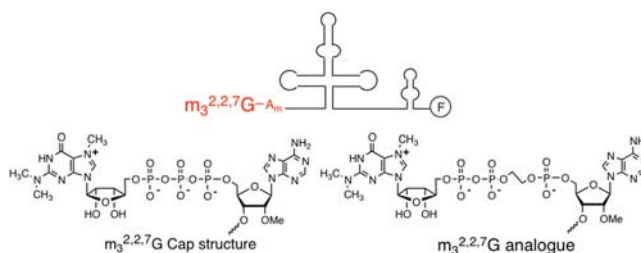


Chemical Synthesis of U1 snRNA
DerivativesAkihiro Ohkubo,^{*,†} Yasushi Kondo,[‡] Makoto Suzuki,[†] Haruki Kobayashi,[†]
Takashi Kanamori,[†] Yoshiaki Masaki,[†] Kohji Seio,[†] Kiyoshi Nagai,[‡] and Mitsuo Sekine^{*,†}Department of Life Science, Tokyo Institute of Technology, 4259 Nagatsuta, Midoriku,
Yokohama 226-8501, Japan, and MRC Laboratory of Molecular Biology, Francis Crick
Avenue, Cambridge Biomedical Campus, Cambridge, CB2 0QH, U.K.

aohkubo@bio.titech.ac.jp; msekine@bio.titech.ac.jp

Received July 8, 2013

ABSTRACT



U1 snRNA is an interesting biological tool for splicing correction and regulation of gene expression. However, U1 snRNA has never been chemically synthesized. In this study, the first chemical synthesis of U1snRNA and its analogues was carried out. Moreover, it was found that the binding affinity of the modified U1 snRNA with an ethylene glycol linkage to snurportin 1 (nuclear import adaptor) was as high as that of the unmodified RNA.

Uridine-rich small nuclear RNAs (U snRNAs) are involved in very important pre-mRNA splicing events in the nucleus of eukaryotic cells.¹ Among them, U1snRNA can recognize the 5'-splice site of pre-mRNA. At the first splicing step, U1snRNA directly binds to the splice site by hybridization between the 5'-terminal eight nucleotides of U1snRNA and the complementary sequence of pre-mRNA.²

Splicing disorders are estimated to account for about 15% of disease-causing mutations in humans, and the majority of genetic mutations are point mutations within the sequences of splice sites.³ A subset of these defects should result from mismatched base pairs between U1snRNA and mutant pre-mRNAs. It was reported in a recent study that mutant pre-mRNA splicing could be

promoted by modified U1snRNAs that were prepared using the biological technique in the cell and could hybridize with the mutant pre-mRNA.⁴ These results indicate that modified U1snRNA could be useful for splicing correction in gene therapy.

Additionally, a modified U1snRNA containing the sequence complementary to the 3'-terminal exon of the targeted gene can downregulate the targeted gene expression via U1 interference (U1i).⁵ This downregulation results from inhibition of mRNA polyadenylation by the U1 snRNA-binding protein (U1–70K protein). However, U1 snRNA has never been chemically synthesized because U1 snRNA is very long (164 nt) and contains a complicated 5'-terminal 2,2,7-*N*-trimethylguanosine ($m_3^{2,2,7}G$)

[†] Tokyo Institute of Technology.[‡] MRC Laboratory of Molecular Biology.(1) (a) Jurica, M. S.; Moore, M. J. *Mol. Cell* **2003**, *12*, 5–14. (b) Wahl, M. C.; Will, C. L.; Lührmann, R. *Cell* **2009**, *136*, 701–718.(2) Pomeranz Krummel, D. A.; Oubridge, C.; Leung, A. K. W.; Li, J.; Nagai, K. *Nature* **2009**, *458*, 475–481.(3) Sironi, M.; Pozzoli, U.; Cagliani, R.; Comi, G. P.; Bardoni, A.; Bresolin, N. *Hum. Genet.* **2001**, *109*, 73–84.(4) (a) Roca, X.; Krainer, A. R. *Nat. Struct. Mol. Biol.* **2009**, *16*, 176–182. (b) Alanis, E. F.; Pinotti, M.; Mas, A. D.; Balestra, D.; Cavallari, N.; Rogalska, M. E.; Bernardi, F.; Pagani, F. *Hum. Mol. Genet.* **2012**, *21*, 2389–2398.(5) (a) Abad, X.; Vera, M.; Jung, S. P.; Oswald, E.; Romero, I.; Amin, V.; Fortes, P.; Gunderson, S. I. *Nucleic Acids Res.* **2008**, *36*, 2338–2352. (b) Grünweller, A.; Hartmann, R. K. *ChemBioChem* **2009**, *10*, 1599–1601. (c) Kanoepfel, A. S.; Abad, A.; Abad, X.; Fortes, P.; Berkhout, B. *Antiviral Res.* **2012**, *94*, 208–216.(6) (a) Pitsch, S.; Weiss, P. A.; Jenny, L.; Stutz, A.; Wu, X. *Helv. Chim. Acta* **2001**, *84*, 3773–3795. (b) Shiba, Y.; Masuda, H.; Watanabe, N.; Ego, T.; Takagaki, K.; Ishiyama, K.; Ohgi, T.; Yano, J. *Nucleic Acids Res.* **2007**, *35*, 3287–3296. (c) Lang, K.; Micura, R. *Nat. Protoc.* **2008**, *3*, 1457–1466. (d) Huber, J.; Cronshagen, U.; Kadokura, M.; Marashally, C.; Wada, T.; Sekine, M.; Lührmann, R. *EMBO J.* **1998**, *17*, 4114–4126. (e) Strasser, A.; Dickmanns, A.; Lührmann, R.; Ficner, R. *EMBO J.* **2005**, *24*, 2235–2243.

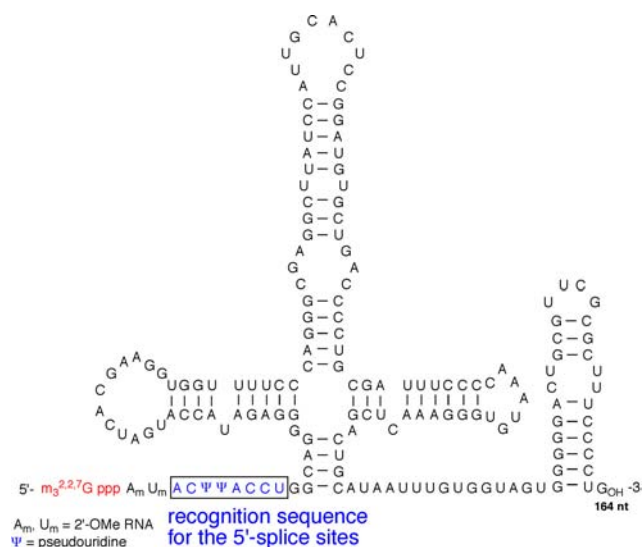


Figure 1. Structure of U1snRNA.

cap (Figures 1 and 2a).⁶ The $m_3^{2,2,7}$ G cap can bind to the nuclear import adaptor snurportin 1 (SPN1) and plays an important role in transporting UsnRNAs between the cytoplasm and the nucleus. In this study, we carried out the chemical synthesis of U1snRNA and modified U1snRNAs containing the $m_3^{2,2,7}$ G cap analogues for the first time, as shown in Figure 2.

Figure 3 depicts the strategy for synthesizing U1snRNA with a fluorescent label at the 3'-terminus. First, we synthesized 5'-short RNA containing the $m_3^{2,2,7}$ G cap by chemical reactions on polymer supports (Schemes 1 and 2) and 3'-long RNA by biochemical methods using T7 RNA polymerase and a chemical reaction in solution phase (Scheme 3). We subsequently generated the complex formed between 5'-short RNA, 3'-long RNA, and the complementary splint DNA. After hybridization, the ligation reactions were carried out using T4 DNA ligase.⁷

In the synthesis of 5'-short RNAs, we used the highly cross-linked polystyrene resin⁸ **1** that has a silyl-type linker,⁹ which can be cleaved under neutral conditions, phosphoryl¹⁰ and phosphityl reagents **2–4** (Figure 4). The synthesis of 5'-short RNA **I** is shown in Scheme 1. After chain elongation of the RNA sequence using 2'-*O*-TBDMS RNA phosphoramidite units on the resin by using an RNA synthesizer, the treatment of resin **5** with 100 equiv of diphenyl phosphonate in pyridine was followed by hydrolysis mediated by a *H*-phosphonate

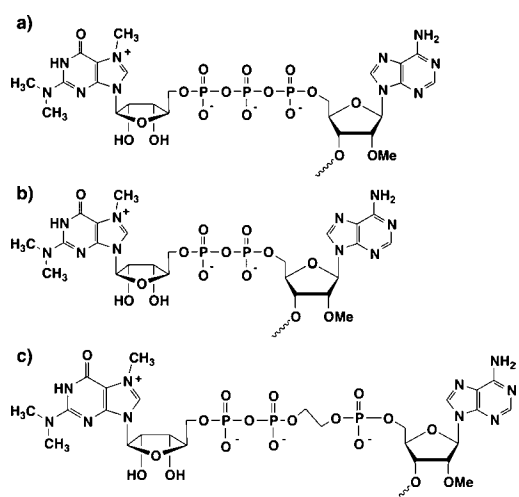


Figure 2. Chemical structure of (a) $m_3^{2,2,7}$ G cap, (b) pyrophosphate analogue, and (c) ethylene glycol analogue.

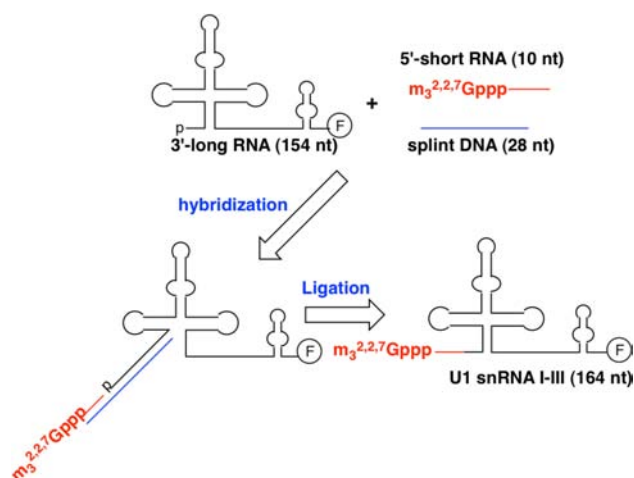


Figure 3. Strategy for U1snRNA synthesis.

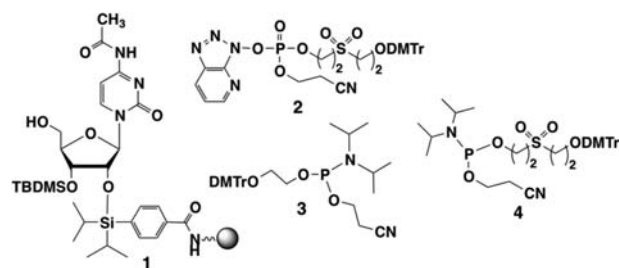
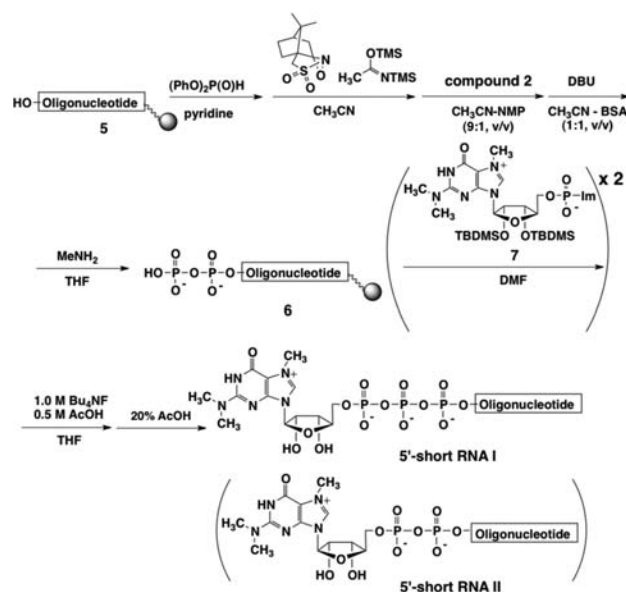


Figure 4. Structure of resin **1**, phosphoryl and phosphityl reagents **2–4**.

- (7) Moore, M. J.; Sharp, P. A. *Science* **1992**, *256*, 992–997.
 (8) McCollum, C.; Andrus, A. *Tetrahedron Lett.* **1991**, *32*, 4069–4072.
 (9) (a) Ohkubo, A.; Seio, K.; Sekine, M. *J. Am. Chem. Soc.* **2004**, *126*, 10884–10896. (b) Ohkubo, A.; Kasuya, R.; Aoki, K.; Kobori, A.; Taguchi, H.; Seio, K.; Sekine, M. *Bioorg. Med. Chem.* **2008**, *16*, 5345–5351.
 (10) (a) Ohkubo, A.; Aoki, K.; Seio, K.; Sekine, M. *Tetrahedron Lett.* **2004**, *45*, 979–982. (b) Ohkubo, A.; Tago, N.; Yokouchi, A.; Nishino, Y.; Yamada, K.; Tsunoda, H.; Seio, K.; Sekine, M. *Org. Lett.* **2012**, *14*, 10–13.

compound. The compound was subsequently oxidized using (2*R*,8*S*)-(+)-(camphorsulfonyl)oxaziridine in the presence of *N*,*O*-bis(trimethylsilyl)acetamide (BSA). The 5'-terminal pyrophosphate bond was formed by the reaction of the protected oligonucleotide immobilized on the resin with compound **2** for 15 min, as shown in Scheme 1. After deprotection of the 2-cyanoethyl, sulfonylethyl, and *N*-acyl groups, the 5'-terminal capping reaction was performed by treating residue **6** with $m_3^{2,2,7}$ G phosphorimidazolide unit **7**.¹¹ The 2'-TBDMS groups of the oligomer were subsequently deprotected, and the oligomer was released from the resin by treatment with 1 M tetrabutylammonium fluoride (TBAF) in the presence of 0.5 M AcOH for 24 h. Although the only 2'-TBDMS groups of pseudouridine residues could not be cleaved under the deprotection conditions using TBAF, the TBDMS groups were easily removed by treatment with a 20% acetic acid aqueous solution for 3 h.¹² The target 5'-short RNA **I** was isolated by anion-exchange HPLC in 2% yield and characterized by MALDI-TOF mass spectrometry. Additionally, the 5'-short RNA **II** having a pyrophosphate group was obtained as a byproduct in 3% isolated yield.

Scheme 1. Synthesis of 5'-Short RNA **I** Containing an $m_3^{2,2,7}$ G Cap^a

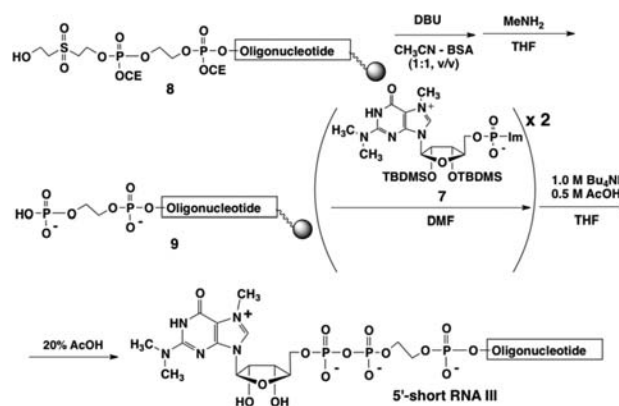


^a The sequence of oligonucleotide is $A_mU_mAC\Psi\Psi AC$.

In the synthesis of 5'-short RNA **III** containing an $m_3^{2,2,7}$ G cap analogue, the resin **8** was prepared by chain elongation using phosphityl reagents **3** and **4** on resin **1**. Resin **8** was treated with DBU and MeNH₂ to obtain resin **9**. After capping using phosphorimidazolide unit **7**, the oligomer was deprotected and released from the resin in 12% isolated yield.

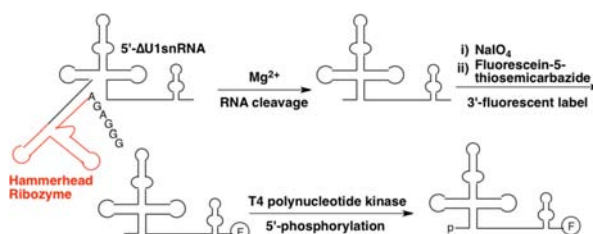
The yield of 5'-short RNA **III** was much higher than that of RNA with an unmodified $m_3^{2,2,7}$ G cap.

Scheme 2. Synthesis of 5'-Short RNA **III** Containing an $m_3^{2,2,7}$ G Cap Analogue^a



^a The oligonucleotide sequence is $A_mU_mAC\Psi\Psi AC$.

Scheme 3. Synthesis of 3'-Long RNA



The 3'-long RNA was synthesized by RNA transcription using T7 RNA polymerase, as shown in Scheme 3. In this RNA transcription, we combined the desired RNA sequence and the hammerhead ribozyme sequence because efficient synthesis requires the RNA to have a purine-rich sequence at the 5'-terminus.¹³ The 5'-sequence of hammerhead ribozyme was efficiently eliminated under the transcription conditions in the presence of Mg²⁺. After PAGE purification, we introduced a fluorescent label into the 3'-terminus of the RNA by treatment with NaIO₄ and fluorescein-5-thiosemicarbazide in 65% isolated yield. For the final step, 5'-phosphorylation was carried out using T4 polynucleotide kinase.

The 5'-short RNAs **I–III** were ligated with the 3'-long RNA by T4 DNA ligase to obtain the U1 snRNA **I** and its analogues (U1 snRNA **2** with a pyrophosphate linkage and U1 snRNA **III** with an ethylene glycol linkage) with a fluorescent label in the presence of the complementary splint DNA. The target U1 snRNAs **I–III** were isolated by PAGE in 14, 28, and 30% yields, respectively, and characterized by MALDI-TOF mass spectrometry. These

(11) Ohkubo, A.; Sasaki, K.; Noma, Y.; Tsunoda, H.; Seio, K.; Sekine, M. *Bioorg. Med. Chem.* **2009**, *17*, 4819–4824.

(12) Kawahara, S.; Wada, T.; Sekine, M. *J. Am. Chem. Soc.* **1996**, *118*, 9461–9468.

(13) Price, S. R.; Ito, N.; Oubridge, C.; Avis, J. M.; Nagai, K. *J. Mol. Biol.* **1995**, *249*, 398–408.

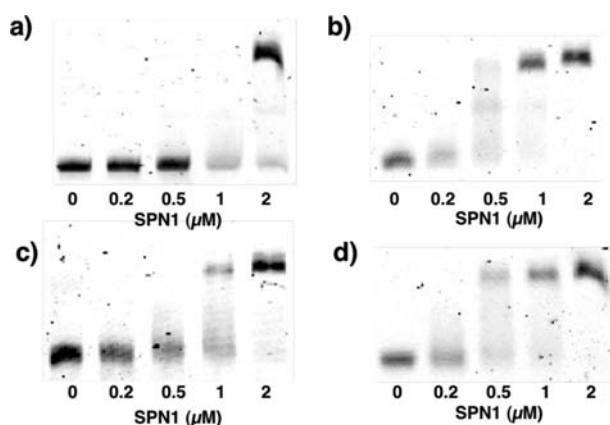


Figure 5. Electrophoretic mobility shift assay of the complexes formed between snurportin 1 (0–2 μ M) and U1 snRNA I–III (50 nM) on a 16% nondenaturing polyacrylamide gel at pH 7.4. (a) U1snRNA without an $m_3^{2,2,7}$ G cap, (b) unmodified U1 snRNA I, (c) modified U1 snRNA II, and (d) modified U1 snRNA III with an ethylene glycol linkage.

results are the first demonstration of the chemical synthesis of U1 snRNA and their analogues.

Moreover, we examined the binding affinities of U1 snRNA I–III to the nuclear import adaptor SPN 1, as shown in Figure 5. The RNA without an $m_3^{2,2,7}$ G cap could not specifically bind to SPN 1, though nonspecific binding was observed in 2 μ M SPN1 due to electrostatic interaction between the phosphate groups of the RNA and lysine (or arginine) residues of SPN1 (Figure 5a). On the other hand, U1 snRNA I with an $m_3^{2,2,7}$ G cap could completely bind to SPN1 in 1 μ M SPN1 in Figure 5b. Figure 5c and 5d show the results of U1snRNAs with $m_3^{2,2,7}$ G cap analogues. Interestingly, the binding affinity

of the analogue with an ethylene glycol linkage was as high as that of U1 snRNA I with an $m_3^{2,2,7}$ G cap, as shown in Figure 5d, though the binding affinity of the U1 snRNA II with a pyrophosphate linkage was lower than that of U1 snRNA I. Ficner et al. previously reported that the $m_3^{2,2,7}$ G interacts with another nucleobase and Trp276 of SPN1 in the crystal structure of the complex formed between SPN 1 and the $m_3^{2,2,7}$ GpppG dimer.^{6e} Therefore, the shortage of the atom length of the pyrophosphate linkage in U1 snRNA II might decrease the binding affinity to SPN 1. On the other hand, a longer linkage such as that afforded by ethylene glycol in U1 snRNA III might not affect the binding affinity to SPN 1.

In summary, we carried out a first chemical synthesis of U1snRNA and its analogues by a combination of solid-phase and enzymatic reactions. The synthesis of U1 snRNA III with an $m_3^{2,2,7}$ G cap analogue was easier and more efficient than that of U1 snRNA I with an $m_3^{2,2,7}$ G cap. Moreover, we found that the binding affinity of U1 snRNA III was as high as that of U1snRNA I. These results should be very useful for investigating splicing correction and U1i by an artificial U1 snRNA in gene therapy. Further studies on these issues are currently in progress.

Acknowledgment. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and Izumi Science and Technology Foundation.

Supporting Information Available. Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.