'Waltz of the polypeptides' by Mara G. Haseltine, Cold Spring Harbor Laboratory, NY, USA. Credit: Ivanka Kamenova / Springer Nature Limited

MILESTONES

MILESTONE 1

First signs of antisense RNA activity

The first reports of antisense RNA activity in eukaryotes were published when the efforts to understand the mechanism of mRNA translation were well underway. At that time, cell-free in vitro translation systems were widely used to probe the function of different translation co-factors. These minimal systems, comprised of a cell extract and a template mRNA, proved instrumental in the initial characterisation of cellular RNAs that could control mRNA translation through antisense recognition of their targets.

Working in such a cell-free system, in 1975, Stuart Heywood and colleagues demonstrated that short ribonucleotide sequences purified from chicken muscle messenger ribonucleoprotein and polysome fractions could control translation of the mRNA encoding myosin. They called the RNAs 'translation control RNAs' (tcRNAs) and proposed that tcRNAs act by binding to their mRNA targets in a sequence-specific manner. In follow-up work a decade later, Heywood demonstrated that one of these tcRNAs, tcRNA102, recognises a sequence in the 5′ untranslated region of chicken myosin mRNA, albeit with imperfect homology.

In the years immediately following Heywood's original tcRNA discovery, several groups isolated similar small RNA species from different organisms and demonstrated that these short RNAs could modulate translation. Notably, in 1977, Severo Ochoa and colleagues purified two distinct short RNAs from a small crustacean, *Artemia salina*, and showed that these RNAs exerted activating and inhibitory effects on *A. salina* mRNAs. The activator RNA is complementary to the inhibitory RNA, and thus its stimulatory effect on translation was proposed to be due to sequestering the inhibitory RNA through base-pairing. The ideas put forward by Ochoa — that small regulatory RNAs exist as double stranded structures and are generated by endogenous RNase enzymes — have been revisited in subsequent decades, as the mechanisms for processing of small interfering RNAs and microRNAs were uncovered.

Whereas the above reports described the activity of cellular antisense RNAs, in 1977, Paterson et al. demonstrated that exogenous plasmid DNA fragments complementary to an mRNA could also inhibit its translation in vitro. A year later, Paul Zamecnik and Mary Stephenson reported the first synthetic DNA oligonucleotide delivered to cells, capable of inhibiting viral replication and oncogenic transformation caused by Rous sarcoma virus. The 35S RNA of this retrovirus had recently been found to contain a 20-nucleotide repeat sequence in its 5′ and 3′ ends. The authors synthesized 13-nucleotide-long DNA oligonucleotides complementary to part of the viral repeat sequence, which was hypothesized to be important for viral replication, and tested their effects on the growth of chicken embryonic fibroblasts infected with Rous sarcoma virus. Two synthetic oligonucleotide variants were tested: one with free 3′ and 5′ termini, and one with chemical modifications. Strikingly, addition of either oligonucleotide to the cell culture medium at the time of infection inhibited viral replication and oncogenic transformation of the cells. The chemically modified oligonucleotide performed better, likely because the modifications conferred resistance to nucleases in the culture medium.

The most likely target processes of the complementary oligonucleotide were the circularisation of provirus DNA or the translation of viral mRNA. Although the first possibility was not ruled out, in a companion paper published in the same issue of *Proceedings of the National Academy of Sciences*, Stephenson and Zamecnik demonstrated that the delivered oligo inhibits translation of viral mRNA through sequence-specific hybridisation.

Our knowledge of the mechanisms by which endogenous RNAs regulate key processes in cell function and in development has since grown exponentially. Yet, these early experiments, using highly purified components, provided the first hints that cells produce small RNAs with the capacity to affect translation, and that the complementarity of these RNAs to their target is important for their function. The experiments by Zamecnik, Stephenson and others laid the foundation of the field of antisense RNA therapeutics and resulted in the founding of Hybridon, the first biotechnology company dedicated to developing synthetic oligonucleotides for therapeutic purposes.

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MILESTONE STUDIES Bester, A. J. et al. Two classes of translational control RNA: their role in the regulation of protein synthesis. *Proc. Natl Acad. Sci. USA* **72**, 1523–1527 (1975) | Lee-Huang, S. et al. Eucaryotic oligonucleotides affecting mRNA translation. *Arch. Biochem. Biophys.* **180**, 276–287 (1977) | Paterson, B. M. et al. Structural gene identification and mapping by DNA–mRNA hybrid-arrested cell-free translation. *Proc. Natl Acad. Sci. USA* **74**, 4370–4374 (1977) | Zamecnik, P. C. et al. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl Acad. Sci. USA* **72**, 280–284 (1978) | Stephenson, M. L. et al. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc. Natl Acad. Sci. USA* **75**, 285–288 (1978).
FURTHER READING Heywood, S. M. tcRNA as a naturally occurring antisense RNA in eukaryotes. *Nucleic Acids Res.* **14**, 6771–6772 (1986) | Zamecnik, P. C. From protein synthesis to genetic insertion. *Annu. Rev. Biochem.* **74**, 1–28 (2005) | Kresge, N. et al. The Discovery of tRNA by Paul C. Zamecnik. *J. Biol. Chem.* **280**, e37 (2005).