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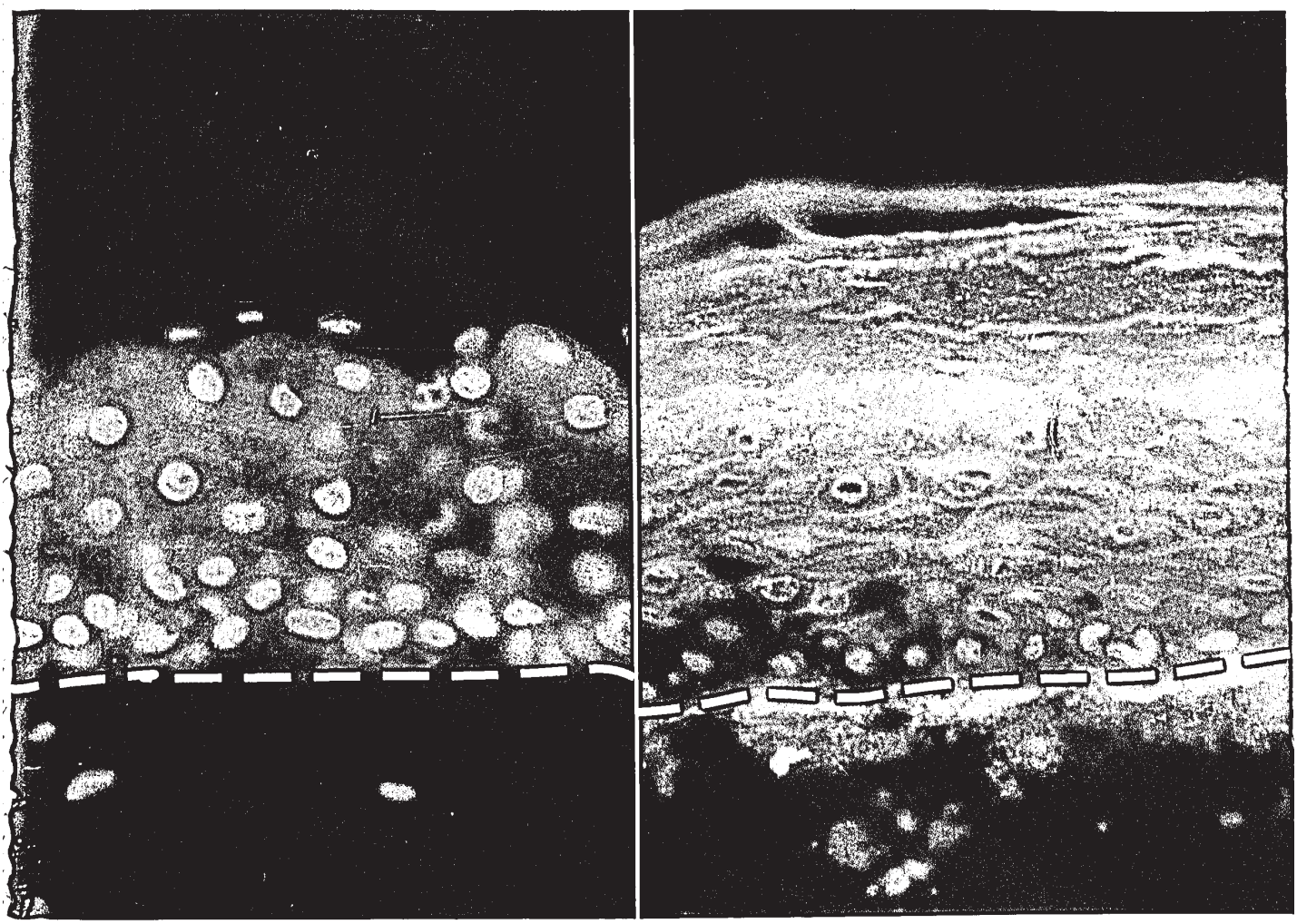

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Molecular Therapy is the monthly publication of the American Society of Gene Therapy (ASGT). The journal publishes original scientific papers in the areas of gene transfer, gene regulation, gene discovery, cell therapy, experimental models, correction of genetic and acquired diseases, and clinical trials. Manuscripts describing new methodological advances will also be considered for publication. In addition, *Molecular Therapy* publishes timely reviews, commentaries, and scientific correspondence. Although *Molecular Therapy* is the official journal of ASGT, it is international in scope and publication. The major criteria for acceptance and publication of a manuscript are originality, high quality, scientific rigor, and interest to a wide audience of readers.

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All correspondence should be addressed to: Robert Frederickson PhD, Editor for *Molecular Therapy*, 214 Summit Avenue East, Suite 305, Seattle, WA 98102-5640. Tel/Fax: 206-724-7760. Email: editor@molther.org. All manuscripts should be submitted online at: https://www.editorialmanager.com/mthe/.

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All business correspondence and inquiries should be addressed to: *Molecular Therapy*, Nature Publishing Group, 25 First Street, Suite 104, Cambridge, MA 02141. Tel: +1 617 475 9221. Fax: +1 617 494 4960.

Publishing Manager: Elizabeth Durzy Senior Production Editor: Anthony Dunlap
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For information, contact the American Society of Gene Therapy at:
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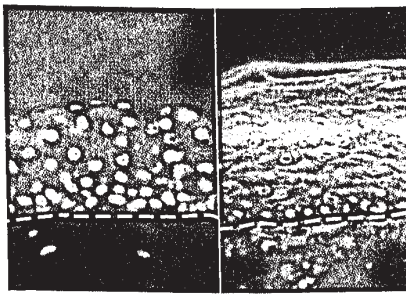

On the cover:

Inhibition of multidrug-resistant *Acinetobacter baumannii* by non-viral expression of hCAP-18 in a bioengineered human skin tissue. See the article by Thomas-Virnig *et al.* on pages 562–569.

Molecular Therapy

Guidelines for Antisense Oligonucleotide Design and Insight Into Splice-modulating Mechanisms

Annemieke Aartsma-Rus[1], Laura van Vliet[1], Marscha Hirschi[1], Anneke AM Janson[2], Hans Heemskerk[1], Christa L de Winter[1], Sjef de Kimpe[2], Judith CT van Deutekom[2], Peter AC 't Hoen[1] and Gert-Jan B van Ommen[1]

[1]Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands; [2]Prosensa Therapeutics B.V., Leiden, The Netherlands

Antisense oligonucleotides (AONs) can interfere with mRNA processing through RNase H–mediated degradation, translational arrest, or modulation of splicing. The antisense approach relies on AONs to efficiently bind to target sequences and depends on AON length, sequence content, secondary structure, thermodynamic properties, and target accessibility. We here performed a retrospective analysis of a series of 156 AONs (104 effective, 52 ineffective) previously designed and evaluated for splice modulation of the dystrophin transcript. This showed that the guanine-cytosine content and the binding energies of AON-target and AON–AON complexes were significantly higher for effective AONs. Effective AONs were also located significantly closer to the acceptor splice site (SS). All analyzed AONs are exon-internal and may act through steric hindrance of Ser-Arg-rich (SR) proteins to exonic splicing enhancer (ESE) sites. Indeed, effective AONs were significantly enriched for ESEs predicted by ESE software programs, except for predicted binding sites of SR protein Tra2β, which were significantly enriched in ineffective AONs. These findings compile guidelines for development of AONs and provide more insight into the mechanism of antisense-mediated exon skipping. On the basis of only four parameters, we could correctly classify 79% of all AONs as effective or ineffective, suggesting these parameters can be used to more optimally design splice-modulating AONs.

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INTRODUCTION

Antisense oligonucleotides (AONs) are useful tools to modulate gene expression in a sequence-specific manner (reviewed in ref. 1). Generally, AONs are used to induce gene knockdown through RNase H cleavage of DNA:RNA hybrids of an mRNA. In addition, mRNA translation can be arrested by steric hindrance of the ribosomal complex by the AON. Finally, AONs can interfere with the splicing process to induce nonfunctional mRNAs that are subjected to the nonsense-mediated RNA decay pathway. Using the latter approach, it is also feasible to modulate alternative splicing, or to block aberrant, disease-causing splice sites (SSs).[2] These mechanisms can be used for studies on developmental processes by allowing knockdown of genes at specific time points,[3] or for therapeutic purposes. In fact, an RNase H–inducing AON is registered under the name Vitravene to treat cytomegaloviral-induced retinitis, and many AONs aiming at targeted gene downregulation are in late stage clinical trials mainly as putative anticancer drugs.[1] Splice-modulating AONs are in early phase clinical trials for Duchenne muscular dystrophy (DMD).[4] Here, the modulation of splicing (in this case the skipping of an exon) aims to restore the disrupted dystrophin-reading frame, allowing the generation of partly functional proteins and slowing down the severe, progressive muscle wasting phenotype.

Each antisense mechanism requires stable and efficient binding of the AON to its target sequence. One obvious determinant of AON efficacy is the accessibility of the target (**Supplementary Figure S1**). Several software programs are available to predict the secondary structure of RNA, of which the m-fold server is the most widely used.[5] This server also provides a so-called SS-count for the target sequence, indicating the propensity of a nucleotide to be single stranded in a number of potential secondary structure predictions. This approach probably reflects the actual *in vivo* situation more closely than focusing only on the most energetically stable structure. In addition, the stability and binding energy of the AON to the target sequence influence AON efficiency. This depends on *e.g.*, AON length and sequence constitution and the free energy of local structures.[1] To efficiently bind a target sequence, the free energy of the AON-target complex must be higher than that of the target complex and that of the AON. As AONs are generally only 17–25-nucleotides long, they are unlikely to form stable secondary structures. However, most AONs can form AON–AON complexes with other AONs of the same sequence (**Supplementary Figure S2**). The software program RNAstructure 4.5 has a tool that provides the free energy of AON–AON complexes and AON-target complexes, in addition to the free energy of individual AONs and the target sequence.[6] The aforementioned software programs (as well as others) can be used to facilitate AON design (reviewed in ref. 1). Nonetheless, none of them is 100% conclusive or predictive and in general a trial and error procedure is still involved to identify potent AONs.

Correspondence: *Annemieke Aartsma-Rus, Department of Human Genetics, Leiden University Medical Center, PO Box 9600, Postzone S4-P, 2300 RC, Leiden, The Netherlands. E-mail: a.m.rus@lumc.nl*

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