

METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

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RNA Remodeling Proteins

Methods and Protocols

Edited by

Marc Boudvillain

CNRS Centre de Biophysique Moléculaire, Orléans, France

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Editor

Marc Boudvillain
CNRS Centre de Biophysique Moléculaire
Orléans, France

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Preface

The purpose of this book is to provide the scientific community with current methods to study RNA remodeling proteins. With the continuous discovery of new classes of functional RNAs comes the understanding that the role and fate of RNA species in cells is intimately linked to timely adjustments of their structures and interactions with other biomolecules. Nascent RNA transcripts may fold spontaneously as they emerge from the transcription machinery, but their maturation into germane structures (alone or in complexes with proteins) as they are transported, utilized, or recycled within cells requires specialized proteins. Several classes of proteins contribute to remodel the structure of RNA and RNA–protein complexes. RNA helicases, which convert the energy derived from the binding and/or hydrolysis of a NTP cofactor into mechanical work, constitute a major class of RNA remodeling proteins. Other important classes include RNA-binding proteins that reduce the RNA conformational space and/or accelerate RNA structure formation, RNA chaperones that first disrupt double-stranded regions upon their binding to RNA (and then also often accelerate RNA structure formation), or enzymes that irreversibly alter RNA structure such as exoribonucleases. The field thus has to deal with a great diversity of proteins and mechanisms (selected representatives are presented in this book) but ultimately thrives from a common bulk of expertise that includes state-of-the-art molecular genetics, enzymology, protein and RNA biochemistry, and biophysics techniques. In the chapters of this book written by leading international experts, the reader will find procedures to identify RNA remodeling proteins and their cofactors, uncover their physiological RNA targets and biological functions, or unravel their complex molecular mechanisms of action using purified components.

The book starts with two overview chapters that are intended primarily for readers new to the field, yet constitute healthy reminders to others. Chapter 1 provides basic concepts and simple rules that one needs to follow to study RNA remodeling proteins in the most appropriate way. Chapter 2 is a historical perspective on DEAD-box proteins (which compose one of the most important and abundant groups of RNA remodeling proteins) and the methods that have been pioneered to unravel their biological functions and mechanisms and that have often been proven useful to study other classes of RNA binding proteins.

RNA–protein complexes are often highly dynamic entities, and one of the most challenging tasks consists in the identification of functionally relevant RNA and protein partners. Popular methods rely on high-throughput approaches involving characterization by mass spectrometry (MS) and/or next-generation sequencing (NGS). Chapter 3 contains protocols for the identification of proteins that directly bind to messenger RNAs (mRNAs) through MS analysis of the protein contents of polyA(+) mRNA complexes that have been isolated by *in vivo* UV cross-linking followed by affinity pulldown. Related methods relying on MS identification of proteins that copurify with an RNA remodeling protein of interest—RNA helicase Spb4, for instance—are described in Chapter 4. EMOTE, a novel approach that exploits the power of NGS to analyze how ribonucleases shape cellular transcriptomes and to map the 5′-ends of processed RNAs on a global scale, is presented in Chapter 5. Molecular partnership may also be inferred (or supported) by cellular

co-localization or identification of RNA remodeling components in the same supramolecular assemblies, and Chapter 6 describes how to do so by immunofluorescence microscopy.

Before undertaking extensive characterization of the molecular mechanisms of RNA remodeling proteins, it is usually necessary to assess their biological function(s) and RNA remodeling activity *in vivo*. Chapter 7 details genetics approaches that help performing such tasks in *Salmonella* and related bacteria. Chapter 8 describes a simple complementation assay that is convenient to test the RNA chaperone activity of heterologous proteins in *Escherichia coli*.

Characterization of the molecular mechanisms of RNA remodeling proteins relies both on general methods and on specialized assays that are most appropriate for given class(es) of “remodelers,” ATP-dependent helicases for instance. Chapter 9 provides protocols for the preparation and unwinding activity testing of RHAU, an eukaryotic RNA helicase that disrupts RNA quadruplex structures such as that found in human telomerase RNA. Chapter 10 describes how changes in the configuration of the ATPase pocket that occur during the catalytic cycle of an RNA helicase can be probed by ENDOR, a sophisticated electron paramagnetic resonance spectroscopy approach. Chapter 11 includes protocols for the bioinformatics analysis of conserved structural motifs in DEAD-box RNA helicases, for characterization of their ATP hydrolysis activity using a photometric, malachite green assay, and for analysis of their RNA binding and unwinding activities using electrophoretic mobility shift assays (EMSAs). Chapter 12 provides additional protocols to evaluate the effect of a protein cofactor on the RNA unwinding and annealing activities of a DEAD-box protein using EMSAs.

Although easy and robust, discontinuous EMSAs sometimes lack sufficient precision to extract meaningful reaction parameters. Chapter 13 describes a protocol for the continuous measurement of helicase activity, which is based on Fluorescence Resonance Energy Transfer (FRET), and is precise enough to detect the changes in activation enthalpies that are associated with species adaptation to cold. Continuous FRET-based assays also constitute an interesting platform to screen chemical libraries in search of inhibitors. Chapter 14 describes such an implementation to target the NS3 helicase of hepatitis virus C (HCV) as well as protocols to characterize the mechanisms of action of NS3 inhibitors.

While some helicases—DEAD-box proteins, for instance—have a limited range of action from their binding site, others move along RNA over long distances, displacing obstacles in their paths. Chapter 15 contains protocols for the characterization of the mechanisms of the NS3 helicase both “in bulk,” using a fluorescence assay, and at the single-molecule level using an optical tweezers setup that permits investigation of NS3 translocation/unwinding with base pair spatial resolution. Chapter 16 describes an alternative magnetic tweezers setup to monitor RNA translocation that has lower resolution but allows the simultaneous monitoring of multiple single molecules at once, thereby facilitating detection of low-frequency events and/or production of accurate subpopulation statistics. In the cases where RNA helicases also translocate along DNA, time-resolved probing with DNA structure-specific chemicals such as KMnO_4 , as described in Chapter 17, produces enzyme “footprints” that report on translocase activity and mechanisms.

Chapter 18 contains protocols for the preparation of the hexameric transcription termination Rho helicase from *Mycobacterium tuberculosis* and for characterization of its ATPase turnover using a photometric phosphate assay and RNA unwinding activity using EMSA. Chapter 19 provides protocols for the characterization of the transcription termination activity of Sen1—the functional homolog of Rho in yeast—from reconstituted, bead-affixed transcription complexes. Chapter 20 describes an *in vitro* transcription assay as well

as transcriptional reporter fusions *in vivo* that are used to study the functional consequences of mRNA leader remodeling induced by the attenuator protein TRAP.

As already noted, fluorescence-based assays are particularly well suited to probe conformational changes within biomolecules. Chapter 21 describes how to perform FRET at the single-molecule level to probe the conformational states of RNA structures and their rearrangements upon recognition by proteins. Chapter 22 includes protocols for the labeling of a hexameric RNA chaperone—Hfq—with fluorescent dyes, for the study of protein oligomerization and RNA binding activity by polyacrylamide gel electrophoresis (PAGE) and fluorescence anisotropy, and for measuring kinetics of RNA duplex formation in the presence of the protein chaperone using a stopped-flow spectrometer. Protocols for the labeling of RNA with a fluorescent dye and the quantitative characterization of the electrostatic and non-electrostatic components of RNA–protein interactions using a fluorescence anisotropy-based salt-titration approach are presented in Chapter 23. Footprinting techniques provide valuable information about the precise site(s) of protein binding in RNA and structural changes induced upon binding; Chapter 24 provides protocols for performing footprinting assays with hydroxyl radicals and ribonucleases.

Ribonucleases form a distinct class of RNA remodeling proteins as they modify RNA structure irreversibly upon cleavage of phosphodiester bonds. Chapter 25 contains protocols for the preparation of eukaryotic exoribonucleases and labeled RNA substrates, their use in the biochemical characterization of exonucleolytic activity, and how to discriminate between *exo*- and *endonucleolytic* activities. What it takes to adapt these methods to the study of nucleases from thermophilic Archaea species is discussed in Chapter 26.

This book is the result of a collective effort, and I would like to thank all authors for their enthusiastic contributions and suggestions. I also would like to thank John Walker—the Series editor—for his guidance throughout the process of assembling and editing the book manuscript as well as colleagues at CNRS for valuable comments and suggestions on book contents.

Orléans, France

Marc Boudvillain

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Contributors

- SORAYA AIT-BARA • *Centre de Biochimie Structurale, CNRS UMR5048, Montpellier, France; INSERM (U1054), Montpellier, France; Universités Montpellier I et II, Montpellier, France*
- NOBUYOSHI AKIMITSU • *Radioisotope Center, University of Tokyo, Tokyo, Japan*
- VÉRONIQUE ARLUISON • *Université Paris Diderot, Sorbonne Paris Cité, Paris, France; Laboratoire Léon Brillouin, CEA-Saclay, Gif-sur-Yvette, France*
- JOSETTE BANROQUES • *Institut de Biologie Physico-chimique, CNRS FRE3630, Université Paris Diderot, Sorbonne Paris Cité, Paris, France*
- THIERRY BIZEBARD • *Institut de Biologie Physico-chimique, CNRS FRE3630, Université Paris Diderot, Sorbonne Paris Cité, Paris, France*
- EVAN P. BOOY • *Department of Chemistry, University of Manitoba, Winnipeg, MB, Canada*
- LIONELLO BOSSI • *Centre de Génétique Moléculaire, CNRS UPR3404, Gif-sur-Yvette, France*
- MARC BOUDVILLAIN • *Centre de Biophysique Moléculaire, CNRS UPR4301, Orléans, France*
- BERTRAND CASTAING • *Centre de Biophysique Moléculaire, CNRS UPR4301, Orléans, France*
- WEI CHENG • *Department of Pharmaceutical Sciences, University of Michigan, Ann Arbor, MI, USA; Department of Biophysics, University of Michigan, Ann Arbor, MI, USA*
- CAROLINE CLERTÉ • *Centre de Biochimie Structurale, CNRS UMR5048, Montpellier, France; INSERM (U1054), Montpellier, France; Universités Montpellier I et II, Montpellier, France*
- BÉATRICE CLOUET-D'ORVAL • *UMR 5100-LMGM, CNRS and Université de Toulouse, Toulouse, France*
- FRANCK COSTE • *Centre de Biophysique Moléculaire, CNRS UPR4301, Orléans, France*
- JESÚS DE LA CRUZ • *Departamento de Genética, Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío, Universidad de Sevilla, Sevilla, Spain*
- FRANÇOIS D'HEYGÈRES • *Centre de Biophysique Moléculaire, CNRS UPR4301, Orléans, France; Ecole doctorale Santé, Sciences Biologiques et Chimie du Vivant (ED 549), Université d'Orléans, Orléans, France*
- KAROLINA DRAZKOWSKA • *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland; Institute of Genetics and Biotechnology, University of Warsaw, Warsaw, Poland*
- MARC DREYFUS • *Institut de Biologie Physico-chimique, CNRS FRE3630, Université Paris Diderot, Sorbonne Paris Cité, Paris, France*
- ANDRZEJ DZIEMBOWSKI • *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland; Institute of Genetics and Biotechnology, University of Warsaw, Warsaw, Poland*
- MICHAEL J. ELLIS • *Department of Biochemistry, University of Western Ontario, London, ON, Canada*
- NARA FIGUEROA-BOSSI • *Centre de Génétique Moléculaire, CNRS UPR3404, Gif-sur-Yvette, France*

- FRANCES FULLER-PACE • *Division of Cancer Research, Jacqui Wood Cancer Centre, University of Dundee, Dundee, UK*
- ATSUSHI FURUTA • *Department of Life Science and Medical Bioscience, Waseda University, Tokyo, Japan; Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan*
- JUAN JOSÉ GARCÍA-GÓMEZ • *Departamento de Genética, Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío, Universidad de Sevilla, Sevilla, Spain*
- VERONIKA GOICHEVA • *Centre de Biochimie Structurale, CNRS UMR5048, Montpellier, France; INSERM (U1054), Montpellier, France; Universités Montpellier I et II, Montpellier, France*
- DANIELLA GOLDFARB • *Department of Chemical Physics, Weizmann Institute of Science, Rehovot, Israel*
- PAUL GOLLNICK • *Department of Biological Sciences, University at Buffalo, Buffalo, NY, USA*
- DAVID B. HANIFORD • *Department of Biochemistry, University of Western Ontario, London, ON, Canada*
- YVES HENRY • *Laboratoire de Biologie Moléculaire Eucaryote, CNRS/Université Toulouse Paul Sabatier, Toulouse, France*
- RYOZO IMAI • *Crop Breeding Research Division, Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Hitsujigaoka, Sapporo, Japan*
- ILIA KAMINKER • *Department of Chemical Physics, Weizmann Institute of Science, Rehovot, Israel*
- MYUNG-HEE KIM • *Crop Breeding Research Division, Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Sapporo, Japan; Center for Plant Aging Research, Institute for Basic Science (IBS), Daegu, Republic of Korea*
- KATARZYNA KOWALSKA • *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland; Institute of Genetics and Biotechnology, University of Warsaw, Warsaw, Poland*
- ANTONINA KRAWCZYK • *Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland; Department of Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands*
- SIMON LEBARON • *Laboratoire de Cristallographie et RMN Biologiques, CNRS/Université Paris Descartes, Paris, France*
- DOMENICO LIBRI • *Centre de Génétique Moléculaire, CNRS UPR3404, Gif sur Yvette, France; Institut Jacques Monod, CNRS UMR7592, Université Paris Diderot, Sorbonne Paris Cité, Paris, France*
- PATRICK LINDER • *Department of Microbiology and Molecular Medicine, University of Geneva, Geneva, Switzerland*
- WAI KIT MA • *Department of Biochemistry, Purdue University, West Lafayette, IN, USA*
- EMMANUEL MARGEAT • *Centre de Biochimie Structurale, CNRS UMR5048, Montpellier, France; INSERM (U1054), Montpellier, France; Universités Montpellier I et II, Montpellier, France*
- NATALIE M. McADAMS • *Department of Biological Sciences, University at Buffalo, Buffalo, NY, USA; Department of Microbiology and Immunology, University at Buffalo, Buffalo, NY, USA*
- SEAN A. MCKENNA • *Department of Chemistry, University of Manitoba, Winnipeg, MB, Canada; Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, MB, Canada*

- EWAN K.S. McRAE • *Department of Chemistry, University of Manitoba, Winnipeg, MB, Canada*
- SARAH F. MITCHELL • *Department of Chemistry and Biochemistry, University of Colorado Boulder, Boulder, CO, USA*
- KARIN MUSIER-FORSYTH • *Department of Chemistry and Biochemistry, Center for Retroviral Research, The Ohio State University, Columbus, OH, USA*
- NAOHIRO NODA • *Department of Life Science and Medical Bioscience, Waseda University, Tokyo, Japan; Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan*
- MARCELO NÖLLMANN • *Centre de Biochimie Structurale, CNRS UMR5048, Montpellier, France; INSERM (U1054), Montpellier, France; Universités Montpellier I et II, Montpellier, France*
- SUBRATA PANJA • *T.C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, MD, USA*
- ROY PARKER • *Department of Chemistry and Biochemistry, University of Colorado Boulder, Boulder, CO, USA; Howard Hughes Medical Institute, University of Colorado Boulder, Boulder, CO, USA*
- DUY KHANH PHUNG • *Centre National de la Recherche Scientifique, UMR 5100-LMGM, CNRS and Université de Toulouse, Toulouse, France*
- ODIL PORRUA • *Centre de Génétique Moléculaire, CNRS UPR3404, Gif sur Yvette, France; Institut Jacques Monod, CNRS UMR7592, Université Paris Diderot, Sorbonne Paris Cité, Paris, France*
- KEVIN D. RANEY • *Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR, USA*
- VERONICA M. RANEY • *Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR, USA*
- PETER REDDER • *Faculty of Medicine, University of Geneva, Geneva, Switzerland*
- KIMBERLY A. REYNOLDS • *Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR, USA*
- JOSEPH A. ROSS • *Department of Biochemistry, University of Western Ontario, London, ON, Canada*
- IOULIA ROUZINA • *Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN, USA*
- RICK RUSSELL • *Department of Molecular Biosciences, Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX, USA*
- TIFFINY RYE-McCURDY • *Department of Chemistry and Biochemistry, Center for Retroviral Research, The Ohio State University, Columbus, OH, USA*
- KAZI ABDUS SALAM • *Radioisotope Center, University of Tokyo, Tokyo, Japan; Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi, Bangladesh*
- DESIRÉE SALAS • *Centre de Biochimie Structurale, CNRS UMR5048, Montpellier, France; INSERM (U1054), Montpellier, France; Universités Montpellier I et II, Montpellier, France*
- ANNIE SCHWARTZ • *Centre de Biophysique Moléculaire, CNRS UPR4301, Orléans, France*
- YUJI SEKIGUCHI • *Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan*
- AZIZ TAGHBALOUT • *Department of Molecular Biology and Biophysics, University of Connecticut Health Center, Farmington, CT, USA*

- HIDENORI TANI • *Research Institute for Environmental Management Technology, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan*
- N. KYLE TANNER • *Institut de Biologie Physico-chimique, CNRS FRE3630, Paris, France; Université Paris Diderot, Sorbonne Paris Cité, Paris, France*
- RAFAL TOMECKI • *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland; Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland*
- ELIZABETH J. TRAN • *Department of Biochemistry, Purdue University, West Lafayette, IN, USA; Purdue Center for Cancer Research, Purdue University, West Lafayette, IN, USA*
- RYAN S. TRUSSLER • *Department of Biochemistry, University of Western Ontario, London, ON, Canada*
- SATOSHI TSUNEDA • *Department of Life Science and Medical Bioscience, Waseda University, Tokyo, Japan*
- W. LUKE WARD • *Department of Molecular Biosciences, Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX, USA*
- SARAH A. WOODSON • *T.C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, MD, USA*