The award of the "Interbrew-Baillet Latour de la Santé – 2002" prize to Robert M. Krug for outstanding contributions to influenza virus research

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Dr. Robert M. Krug, Professor at the Institute for Cellular and Molecular Biology, Department of Molecular Genetics and Microbiology at the University of Texas at Austin (U.S.A.), has been awarded the "Interbrew-Baillet Latour de la Santé – 2002" prize for his research on Influenza virus. Count Alfred de Baillet Latour established the "Fonds Interbrew-Baillet Latour" in 1974, and since 1977 the Foundation has awarded the prize to encourage and reward outstanding scientists for research related to human health. Each year, a specific area of health is chosen and thus highlighted. For 2002, the focus was on Influenza.

Dr. Krug's eminence has come from his fundamental contributions to the understanding of the molecular mechanisms that regulate the replication and transcription of Influenza viruses, the agents responsible for three major pandemics in the 20th century. Over the years, Dr. Krug and his colleagues have deciphered the functions and mechanisms of action of many of the influenza virus proteins, and determined how they are related to virus development in the host. Some of the salient features of this work are outlined here.

In a series of ground-breaking papers Dr. Krug documented the "cap-snatching" mechanism that is required for the synthesis of Influenza virus messenger RNAs (mRNAs). Specifically, he demonstrated that Influenza virus mRNA synthesis (*i.e.* transcription) is initiated by cellular primers: 5´ capped (m^{7}GpppNm-containing) fragments that are derived from newly synthesized host cell RNA polymerase II transcripts (cellular pre-mRNAs) in the nucleus [6]. A cap-dependent endonuclease, which is intrinsic to the Influenza virus polymerase, cleaves cellular pre-mRNAs 10–13 nucleotides from their 5´ ends, and the resulting capped RNA fragments serve as primers to initiate viral mRNA synthesis [2, 14,

15]. The discovery that Influenza virus scavenges cellular pre-mRNAs in the nucleus to supply the primers for its own mRNA synthesis dramatically changed fundamental concepts concerning Influenza virus-specific RNA synthesis and how the virus interacts with the cell. This "cap-snatching" mechanism was subsequently shown to be much more widespread than was originally thought.

In a subsequent series of ingenious experiments, Dr. Krug and his colleagues identified the active sites of the Influenza virus polymerase that are required for the primed initiation of viral mRNA synthesis. Capped primers are produced only after the Influenza virus polymerase, which is composed of the three viral polymerase proteins PB1, PB2 and PA [3], is activated by sequential binding of the proteins to the 5´ and 3´ terminal sequences in virion RNA (vRNA). Binding to the 5´ sequence activates the cap-binding site on the PB2 subunit, and subsequent binding to the 3´ sequence activates the endonuclease site on the PB1 subunit. Consequently, the two active sites that comprise the cap-dependent endonuclease are located on different polymerase subunits. Interaction of the PB2 protein with the 5´ terminal cap of mRNAs is probably similar to that of other cap-binding proteins, and the endonuclease site of the PB1 protein is reminiscent of the active sites of other enzymes that cut polynucleotides to produce 3´-OH ends [7, 8]. These results also revealed a potential target for the development of antiviral chemicals: because binding of the 5´ sequence to a specific amino acid sequence in the PB1 protein is a necessary first step in the activation of the viral polymerase, Dr. Krug proposed that this apparently unique RNA-binding domain may be a highly vulnerable site in the virus.

The elucidation of the surprising mechanism of selectivity by the complex of the three polymerase proteins also comes from Dr. Krug's group. The viral polymerase complex selectively protects the 5´ ends of viral mRNAs but not of cellular pre-mRNAs against cleavage by the viral cap-dependent endonuclease. This was an important discovery because if the 5´ ends of viral mRNAs were also cleaved and used as primers, net synthesis of viral mRNAs would not occur. Viral mRNAs are protected because they contain a common sequence adjacent to the 5´ sequence "snatched" from host cell pre-mRNAs. The viral polymerase complex binds to this common sequence and this activates binding of the complex to the capped viral mRNAs. As a result, the 5´ ends of viral mRNAs are protected against cleavage by the cap-dependent endonuclease of the viral polymerase, whereas cellular pre-mRNAs, which lack this common viral sequence, are not protected [20].

In addition, Dr. Krug showed that binding of the viral polymerase complex also plays an essential role in alternative splicing [18]. By binding to the common viral sequence in the viral M1 mRNA, the complex blocks a strong 5´ splice site close to the 5´ end of M1 mRNA. As a result, the cellular splicing machinery switches to a weaker 5´ splice site, resulting in the production of the mRNA that encodes the essential viral M2 ion channel protein. The utilization of the weak M2 5´ splice site requires activation of the site by interaction with a host cell function, a cellular splicing factor called SF2/ASF, which binds to a specific sequence in M1 mRNA [19].

Fundamental advances in our understanding of the mechanism of replication of Influenza virus vRNA and of the regulation of Influenza virus gene expression during infection have come from Dr. Krug's results. For viral mRNA synthesis, both capped RNA-primed initiation and termination at the polyadenylation site are required. Switching from mRNA synthesis (transcription) to full-length complementary RNA synthesis (replication), requires unprimed initiation of RNA synthesis, and prevention of termination at the

polyadenylation site, 15 to 22 nucleotides from the 5´ end of the vRNA template. Viral nucleocapsid protein molecules mediate such an antitermination event, so that template RNAs are produced and coated with nucleocapsid proteins along their entire length [1]. Subsequent copying of the template RNAs to produce vRNAs, also requires the addition of nucleocapsid proteins to the elongating RNA chains. As a result, newly synthesized vRNAs are in the form of nucleocapsids that can be readily packaged into progeny virus particles.

With his colleagues, Dr. Krug established that Influenza virus infection can be divided into early and late phases of gene expression [17]. During the early phase, the synthesis of specific vRNAs, viral mRNAs and viral proteins are tightly coupled. During the late phase, the rate of synthesis of all viral mRNAs decreases dramatically, whereas the rate of synthesis of all vRNAs remains near maximum. In addition, because viral protein synthesis also continues at maximum levels, viral mRNA and viral protein synthesis are not coupled, indicating that previously synthesized viral mRNAs direct protein synthesis during the late phase.

Other studies by Dr. Krug and his group have paved the way for the elucidation of the multiple functions of the Influenza virus non-structural protein NS1. The NS1 protein, which is encoded by the smallest genomic RNA segment of both *Influenza A virus* and *Influenza B virus*, is designated as non-structural because it is synthesized in large amounts in infected cells, but is not incorporated into virions. The NS1 protein of *Influenza A virus* (NS1A protein) was identified over 30 years ago, but little progress in elucidating its functions was made until the early 1990s, when Dr. Krug's group demonstrated that one of the functions of the NS1A protein is inhibition of the nuclear export of poly(A)-containing cellular mRNAs. The NS1A protein contains two domains: an RNA-binding domain at its amino terminal end whose major target is double-stranded (ds) RNA [12], and an effector domain comprising the rest of the protein [16].

Subsequent studies by Dr. Krug and his collaborators elucidated the intriguing mechanism by which the NS1A protein inhibits the nuclear export of poly(A)-containing cellular mRNAs. They showed that the effector domain of the NS1A protein binds and inhibits the function of two cellular proteins that are required for 3´ end processing of cellular pre-mRNAs [4, 10]. As a result of inhibition of 3´ end processing, cellular mRNAs are not exported from the nucleus after *Influenza A virus* infection, but are degraded in the nucleus [13]. The nuclear export of viral mRNAs is not inhibited by the NS1A protein-mediated inhibition of the cellular 3´ end processing machinery because the poly(A) tails of viral mRNAs are produced by the viral polymerase, and not by the cellular 3´ end processing machinery. Further, by employing reverse genetics to generate mutant Influenza A viruses, they showed that the effector domain binding sites for these cellular 3´ end processing factors are important for Influenza virus replication: a mutation in the effector domain of the NS1A protein that abrogated the binding of the one of the cellular 3´ end processing factors resulted in a greatly attenuated virus [9]. This function of the effector domain of the NSlA protein appears to be a countermeasure against an interferon (IFN)-independent cellular antiviral response that occurs soon after *Influenza A virus* infection, a response that is due to the transcription activation of multiple cellular antiviral genes. These cellular antiviral transcripts are synthesized, but their processing to form mature mRNAs in the cytoplasm is blocked by the viral NS1A protein.

The group of Dr. Krug also succeeded in determining the three-dimensional structure of the RNA-binding domain of the NS1A protein [5, 11]. They showed that this domain

exhibits a novel dimeric six-helical chain fold, which is different from that of any other known RNA-binding protein. Subsequent mutagenesis experiments established that the dimer structure is required for RNA binding [22]. In addition, the results of computer modeling and mutagenesis experiments indicated that the RNA-binding domain of the NS1 protein of *Influenza B virus* (NS1B protein), which is 20 amino acids larger than that of the NS1A protein, probably exhibits a similar dimeric six-helical chain fold.

Further, Dr. Krug demonstrated that the activities of the NS1B protein of *Influenza B virus* differ markedly from the activities of the NS1A protein of *Influenza A virus* [21]. The effector domain of the NS1B protein does not inhibit the 3´ end processing of cellular pre-mRNAs and the nuclear export of cellular mRNAs. Rather, the NS1B protein binds ISG15, a human ubiquitin-like protein that is induced after infection by *Influenza B virus*, and also inhibits the conjugation of ISG15 to its target proteins [23]. As is the case for other ubiquitin-like proteins, ISG15 modification probably does not result in the degradation of its target proteins, but rather modifies their activities. These results suggest that the conjugation of ISG15 to its target proteins is detrimental to the replication of *Influenza B virus* and that inhibition of this conjugation by the NS1B protein is the mechanism by which *Influenza B virus* counters this IFN-independent cellular antiviral response. Dr. Krug's results have thus opened up a new exciting area of Influenza virus research: the identification of the protein targets of ISG15, and the determination of how the resulting modification of these target proteins is detrimental to the replication of *Influenza B virus.*

In summary, Dr. Krug has made innovative and important discoveries that have greatly increased our understanding of the molecular mechanisms of Influenza virus replication. The numerous new concepts he has introduced have had a large impact not only on Influenza virus research, but also on virus and cell research in general. Beyond his contribution to a better understanding of the mechanisms regulating Influenza virus replication, the repercussions of Dr. Krug's research have been far-reaching. A few examples suffice to illustrate this conclusion. "Cap-snatching" which constituted a totally novel and unexpected mechanism of viral RNA transcription, was subsequently shown to be far more general, since it is common to all viruses with segmented single-stranded RNA genomes of negative polarity. Finally, probably one of the major advances achieved through the research conducted by Dr. Krug, was to probe successfully into the interactions between viral and cellular proteins, defining how viral proteins specifically affect cellular proteins and thus antagonize the cell cycle.

Dr. Krug's impact on our knowledge of Influenza virus is paramount. His foresight and his ingenuity have given tremendous impetuous to research in Virology at large. Finally, it seems clear that the numerous discoveries made by Dr. Krug have already led – and will lead – to the development of new therapeutic agents.

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