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POINT OF VIEW

An unexpected role for RNA in the recognition of DNA by the innate immune system

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A central function of our innate immune system is to sense microbial pathogens through the presence of their nucleic acid genomes or their transcriptional or replicative activity. In mammals, a receptor-based system is mainly responsible for the detection of these "non-self" nucleic acids. Tremendous progress has been made in the past years in identifying the host constituents that are required for this intricate task. With regard to the sensing of RNA genome based pathogens by our innate immune system, a picture is emerging that includes certain families of the toll-like receptor family (TLR3, TLR7, TLR8) and the RIG-I like helicases (RIG-I, MDA5 and LGP2). Genetic loss of function studies implicate that the absence of these pathways can lead to a complete lack of recognition of certain RNA viruses. At the same time, intracellular DNA can also trigger potent innate immune responses, yet the players in this field are less clear. We and another group have recently identified a role for RNA polymerase III in the conversion of AT-rich DNA into an RNA ligand that is sensed by the RIG-I pathway. In this review article, we will discuss the mechanisms and implications of this novel pathway.

Key words: innate immunity, virus, type I interferon, RIG-I, dsDNA, dsRNA, RNA polymerase III

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Introduction

The integrity of every living organism is under constant threat of attack from the microorganisms in its immediate environment. To this effect, most organisms have evolved efficient strategies to counteract the mere presence or the transcriptional and replicative activity of foreign genetic material. For example, in both plants and invertebrates, one central mechanism of antiviral defense is RNA interference (RNAi), where viral double-stranded RNA (dsRNA) is targeted by RNase III enzymes of the Dicer family and subsequently cleaved into small dsRNA fragments of 21 nucleotides in length. These RNA fragments are incorporated into the RNA-induced silencing complex and finally serve as a "template" for the highly specific cleavage of virus-encoded RNAs. In nematodes and plants, the antiviral activity of siRNA is further amplified through the secondary production of siRNAs that are generated by RNA-dependent RNA polymerases. In higher organisms, a receptor-based anti-microbial innate immune system seems to have replaced the RNAi-based defense system, yet nucleic acids also play a key role here. Pattern-recognition receptors (PRRs) have evolved to detect the presence of "foreign" genetic material, and, following ligand recognition, these receptors trigger the nuclear translocation of key transcription factors that control the expression of anti-microbial effector genes. In the mammalian system, the main antiviral response is controlled by the production of type I interferons. The expression of this gene family mainly depends on the recognition of virus-derived nucleic acids through PRRs. Ultimately, these cytokines provoke the eradication of the invading pathogen and simultaneously regulate adaptive immune responses (reviewed in ref. 1).

Although several types of PRRs have been identified, the family of TLRs is

the group that has been best characterized so far. TLR3, TLR7, TLR8 and TLR9 participate in antiviral immunity by recognizing dsRNA, ssRNA and CpG-rich DNA in the endosomes of dendritic cells and macrophages, respectively.[2] In addition to TLRs, intracellular receptors also detect virus-associated nucleic acids. One such receptor is the double-stranded RNA-activated protein-kinase PKR, which senses dsRNA.[3] However, the role of PKR in initiating antiviral immunity has remained controversial. In 2004, Fujita and colleagues expanded the repertoire of intracellular PRRs by identifying the DexD/H box Helicase RIG-I as a cell-autonomous sensor of dsRNA arising during virus infection.[4] Following this landmark discovery, the family of antiviral RNA helicases was expanded to RIG-I, MDA5 and LGP2, and this group of proteins is often referred to as RIG-I like helicases (RLH). An overview of currently known nucleic acid sensing pathways is depicted in **Figure 1**.

RNA Recognition by RIG-I-Like Helicases

While the RLHs share a common receptor architecture, they differ in their ligand specificity and, thus, their role in the setting of RNA virus infection. Studies using RIG-I deficient mice have demonstrated that RIG-I is essential for the type I interferon response to the negative-stranded viruses, such as paramyxoviruses, VSV and the influenza virus.[5] MDA5-deficient mice, however, are highly susceptible to infection with Picornaviruses, such as Encephalomyocarditis virus or Theiler's virus, which have positive-sense genomes.

The distinct preference for virus recognition by RIG-I and MDA5 is reflected by their different ligand specificities. In an attempt to characterize the molecular ligand for RIG-I, it was first noticed that long homopolymeric dsRNA (Poly(I:C)) was recognized by MDA5 while RIG-I seemed to detect shorter RNAs.[5] In 2006, we and another group independently observed that a 5'-triphosphate moiety was essential for the activation of RIG-I and that this structure represents the molecular substrate that renders in vitro transcribed RNA immunostimulatory.[6,7] Base modifications, capping at the 5' end of the RNA transcript or dephosphorylation prevented detection by RIG-I. As these modifications naturally occur during the maturation of endogenous RNA transcripts but not in the course of the viral, cytosolic replication processes, the presence of a 5'-triphosphate group marked an important molecular structure that allows the host to discriminate self from "foreign" RNA.

Another hallmark of "foreign" RNA is double-strandedness. While short stretches of dsRNA can be present under physiological conditions, longer dsRNA with complete base pairing is usually not found in non-infected cells. In this regard, it is now well established that MDA5 senses dsRNA species. However, two independent reports have shown that RIG-I also requires dsRNA stretches for its activity.[8,9] Until then, knowledge about the stimulatory elements of RIG-I ligands was mostly drawn from studies with 5'-triphosphate RNA generated by in vitro transcription. Using well-defined, chemically synthesized 5'-triphosphate RNA, Schlee et al. and Schmidt et al. showed that single-stranded 5'-triphosphate RNA was not able to trigger the production of type I interferon via RIG-I. Instead, aberrant dsRNA by-products naturally emerging during the in vitro transcription process were found to be responsible for the activation of RIG-I. These immunologically active triphosphorylated dsRNAs are generated as a result of self-coding intramolecular 3'extension and RNA-template-coded de novo synthesis. A thorough investigation of the characteristics of stimulatory RNAs further revealed that a blunt-end formation at the 5'-triphosphate end yielded maximal activity, while overhangs at the 5'-triphosphate end were not tolerated by RIG-I.[8] Accordingly, base pair mismatches in the first position of the complementary RNA strand (opposite the 5'-triphosphate end) markedly reduced the activity. Thus, both the 5'-triphosphate end and double-stranded, blunt-ended RNA-stretches synergize to activate RIG-I.

Double Stranded DNA Triggers Potent Antiviral Responses

In addition to dsRNA, dsDNA can also serve as a pathogen-associated molecular pattern, and its intracellular recognition triggers very strong type I interferon responses.[10,11] While some signaling molecules of the dsDNA-induced signaling pathway have been identified, the actual receptor systems that trigger these pathways are less clear. A candidate DNA receptor DAI (DNA-dependent activation of interferon regulatory factors) has been considered to be potential DNA sensor. However, DAI-deficient mice produce normal amounts of type I interferon in response to dsDNA.[12-14] In addition, we and three other groups recently characterized AIM2 (Absent In Melanoma 2) as a cytosolic DNA receptor that induces the induction of Interleukin-1β via the inflammasome pathway.[15-18] However, AIM2 is not involved in the production of type I interferon. Furthermore, an issue that has remained controversial is the contribution of RIG-I and its signaling molecule MAVS (also known as IPS-1, Cardif, VISA) to dsDNA-induced type I interferon responses. The initial report that had shown that transfection of dsDNA can induce potent type I IFN responses implicated a requirement for MAVS in this pathway.[10] In this respect, it was speculated that, in analogy to RIG-I and MDA5, the putative dsDNA receptor employed MAVS as a signaling molecule. In this study, AT-rich dsDNA (poly(dA:dT)) was used to induce type I IFN production in HEK 293 cells, and MAVS was silenced using siRNA. Another study went on to implicate RIG-I directly in the recognition of dsDNA. Again, AT-rich dsDNA was used to trigger type I production in a human cell line and the involvement of RIG-I was shown by using a cell line that is devoid of functional RIG-I.[19] However, the theory that RIG-I and MAVS were required for dsDNA recognition was challenged by several reports that showed that MAVS- and RIG-I-deficient mice presented completely normal antiviral responses following dsDNA transfection.[20,21]

Intracellular DNA Induces the Production of a Stimulatory RNA Intermediate

Recently, we and another group were able to reconcile some of the controversies with regard to the involvement of RIG-I and MAVS in dsDNA-induced type I

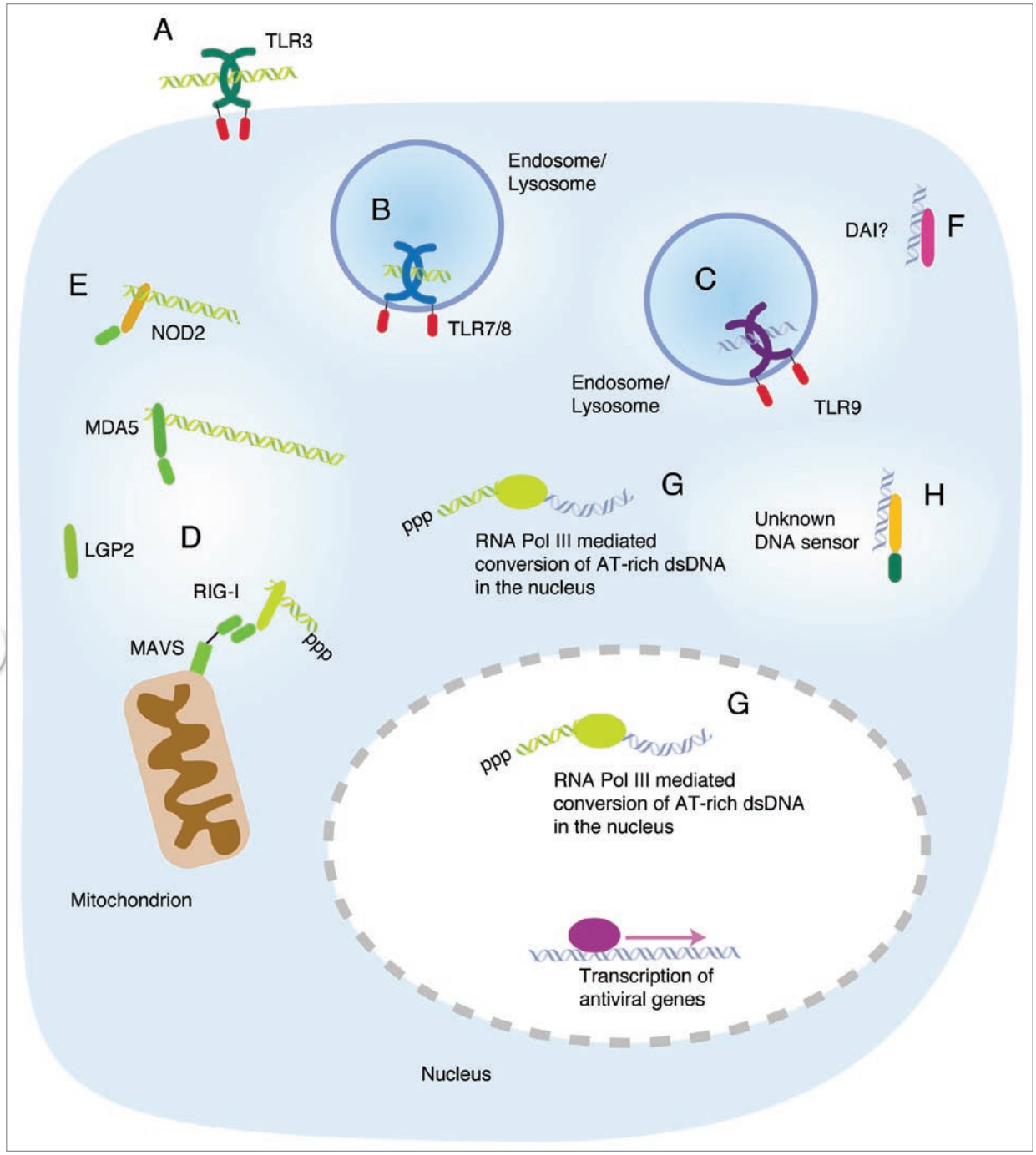

Figure 1. Nucleic acid recognition pathways that lead to activation of central transcription factors in innate immunity. The best studied nucleic acid sensing pathway of the innate immune system is the family of toll-like receptors. Of these, TLR3 (A), located at the cell surface, recognizes dsRNA. TLR7 and TLR8 are confined to endolysosomal compartments, where they sense ssRNA or short dsRNA (B). TLR9, on the other hand, recognizes DNA with a strong preference for sequence motifs containing unmethylated CG dinucleotides (C). In the cytoplasm the family of RIG-I like helicases senses RNA with certain structural features (D). RIG-I is activated by short dsRNA with a 5' triphosphate RNA moiety, whereas MDA5 recognizes long dsRNA. LGP2, which is structurally highly similar to RIG-I and MDA5, yet devoid of a signaling domain, seems to play a modulating role on both RIG-I and MDA5. Next to the RIG-I like helicase family, NOD2 was recently implicated as an additional sensor for cytosolic RNA (E). Cytoplasmic DNA recognition is less well understood. DAI was recently implicated as a cytoplasmic DNA sensor, but studies with DAI-deficient mice suggest that its function is redundant (F). RNA Pol III indirectly contributes to innate immune sensing by transcribing AT rich dsDNA into a RIG-I ligand (G). The exact localization of this pathway, cytoplasm vs. nucleus, is not yet completely solved. In addition, it is very likely that additional sensing pathways exist that contribute to cytoplasmic DNA recognition (H).

interferon production.[22,23] Indeed, we were able to show that beyond its function in the transcription of non-coding RNAs, DNA-dependent RNA Polymerase III (RNA Pol III) participates in DNA-triggered innate immunity by converting AT-rich DNA into a RIG-I stimulatory RNA intermediate. Recognition of this intermediate RNA species via RIG-I then goes on to stimulate the production of interferon.

In an attempt to study the function of different RNA sensors in the recognition of the negative-strand ssRNA virus Sendai Virus (SEV), we unexpectedly observed that the control stimulus, the synthetic dsDNA mimetic poly(dA:dT) induced type I interferon in a RIG-I- and MAVS-dependent fashion. It is important to note that this RIG-I dependency was not observed in primary murine macrophages or bone-marrow-derived dendritic cells, suggesting that in these cell populations at least one other pathway must exist in parallel. As mentioned above, the notion that RIG-I, which is a bona fide RNA sensor, would also respond to dsDNA seemed to be counterintuitive at first sight. Searching for an explanation for this puzzle, we noticed a slight delay in the induction of type I interferon when using dsDNA in comparison to the established RIG-I ligand 5' triphosphate RNA. We thus considered the possibility that poly(dA:dT) might somehow induce an intracellular RNA intermediate that served as a RIG-I activator. The simplest approach to searching for stimulatory RNA intermediates was the isolation of nucleic acids derived from cells transfected with poly(dA:dT) in order to assess their immunostimulatory potential. Indeed, using this approach we found that poly(dA:dT) triggered the de novo synthesis of a stimulatory RNA species. We then tried to ascertain the nature of this RNA ligand by screening a variety of RNA modifying enzymes for their impact on the interferon-inducing activity of this stimulatory RNA. These experiments revealed that transfection of poly(dA:dT) triggered synthesis of a dsRNA intermediate that contained a 5' triphosphate end and was devoid of guanosine. Since such a dsRNA species could consist of adenosines and uracils, we formed the hypothesis that poly(dA:dT) might by transcribed into poly(rA:rU) by a DNA-dependent RNA polymerase.

Transcription by Endogenous DNA-Dependent RNA Polymerases

In eukaryotes, transcription is carried out by three distinct DNA-dependent RNA polymerases, known as RNA Pol I, Pol II and Pol III. These RNA Polymerases are multisubunit enzyme complexes that share a structurally conserved core, yet they differ in the composition of subunits located at the outer part of the transcription complex.[24] Each RNA Pol catalyzes the transcription of a specific set of genes. RNA Pol I synthesizes one large, single precursor ribosomal RNA (rRNA), which is processed into 18S rRNA, 5.8S rRNA and 28S rRNA. In contrast, RNA Pol II-mediated transcription is very complex and includes the synthesis of all protein-encoding mRNAs as well as non-protein coding RNAs (ncRNAs), such as small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and micro RNAs (miRNAs). Conceptually, RNA Pol III more closely resembles RNA Pol I as it is responsible for transcription of ncRNAs. The list of RNAs transcribed by RNA Pol III includes but is not limited to tRNA, 5S rRNA, snRNAs, snoRNAs, miRNAs and Piwi-interacting RNAs.

The primary transcripts of all three RNA Polymerases bear a 5'-triphosphate end as soon as they emerge from the polymerase complex. However, this triphosphate end is often removed or modified by RNA modifying enzymes. For instance, RNA Pol I synthesized precursor-rRNA undergoes multiple exo- and endonucleolytic cleavages, the net result being that ribosomal RNAs bear a 5'-monophosphate group. Primary RNA Pol II transcripts obtain a characteristic 7-methyl-guanosine residue at the 5' end. The addition of this cap occurs during the transcription process, when the nascent transcript encompasses roughly 30 nucleotides and involves the catalytic activity of a RNA-triphosphatase, a guanylyltransferase and a methyltransferase, respectively. Unlike pre-mRNA, RNA Pol III transcripts are synthesized without RNA modifications or processing. Instead, the maturation of RNA Pol III transcripts takes place after synthesis has been completed and the nascent transcript has been released from the transcription complex. Some Pol III transcripts undergo elaborate processing, such as tRNAs, which are nucleolytically processed and finally exhibit a 5'monophosphate. U6 snRNA retains a 5'triphosphate but is O-methylated at the terminal phosphate.[25] The proper modification of this RNA transcript depends on the presence of a conserved stem-loop in a certain sequence context. Thus, transcripts that are artificially transcribed downstream of the U6 promoter (e.g., shRNAs) are not modified at their 5' end.[26] Some natural Pol III transcripts are modified to a lesser extent and some, such as 5S rRNA, retain the 5'-triphosphate terminus of the primary transcript. Since a 5'-triphosphate moiety is a prerequisite for the activation of RIG-I, unmodified RNA Pol III transcripts are per se potential candidates for the source of the stimulatory RNA intermediate.

In retrospect, an intriguing first link between the production of type I interferon and RNA Pol III transcripts had already been described before, when Iggo and colleagues first demonstrated that H1- and U6-promoter driven shRNAs, which are known products of RNA Pol III, are able to trigger type I interferon.[27] Although from their studies it was clear that certain shRNA sequences exhibited strong interferon-inducing effects, the cellular processes underlying this response remained unknown.

Nevertheless, a fundamental difference between RNA Pol III driven shRNA construct and poly(dA:dT) is marked by the absence of a classical promoter-sequence within poly(dA:dT) that would allow specific recruitment of the RNA Pol III transcription machinery. However, homopolymeric poly(dA:dT) sequences are widely used to study promoter-independent transcription. Interestingly, of the three eukaryote RNA polymerases, the preference for poly(dA:dT) as a template has been reported to be highest for RNA Pol III.[28]

Promoter-Independent Transcription of AT-Rich dsDNA by RNA Pol III

On the basis of these indications, we focused our investigations on RNA Pol III.

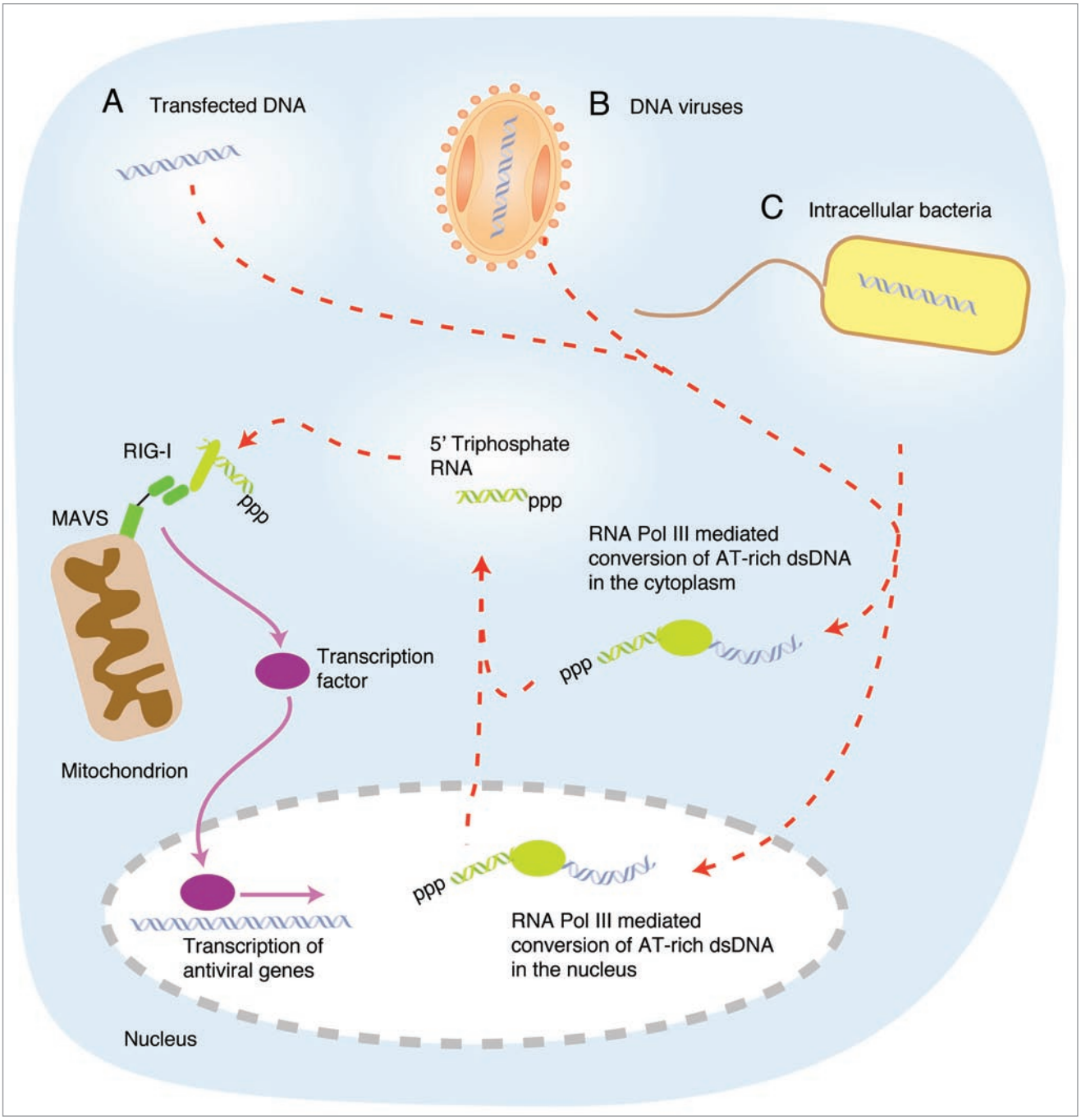

Figure 2. Innate immune sensing by RNA polymerase III. RNA Pol III can transcribe DNA of various sources in a promoter-dependent or promoter-independent fashion. (A) AT-rich DNA, e.g., poly(dA:dT), when transfected into certain cells is transcribed by RNA Pol III in a promoter-independent fashion. The resulting RNA transcript poly(rA:rU) that activates RIG-I has been identified by direct an indirect methods. In addition, synthetic RNA Pol III genes such as shRNAs that are transcribed in a promoter-dependent manner can yield RNA transcripts that are highly RIG-I stimulatory. (B) A similar scenario holds true for Epstein-Barr-Virus, where virus encoded RNA Pol III transcripts are sensed by RIG-I. Moreover, Chiu et al. have shown that HSV-1, that doesn't harbor known RNA Pol III transcribed genes, triggers type I IFN in an RNA Pol III dependent fashion. (C) Intracellularly replicating bacteria, such as *Legionella pneumophila*, have also been reported to trigger the RNA Pol III—RIG-I pathway for type I IFN induction by Chiu and colleagues. Important questions that remain unanswered for these observations are the localization of the RNA Pol III-mediated transcription and the nature of the RNA transcripts being made.

We observed that AT-rich DNA sequences are converted in vivo into poly(rA:rU) transcripts and that RNA Pol III is responsible for this effect. Remarkably, while studying the contribution of single RNA Pol III subunits, we found that two subunits, namely RPC3 and RPC7 were dispensable for de novo synthesis of poly(rA:rU) from the cognate DNA template. Due to extensive studies in the model organism *Saccharomyces cerevisiae*, there is a detailed understanding of the subunits that together form the

RNA Pol III transcription complex. Of the 17 RNA Pol III subunits, 10 are unique to RNA Pol III (RPC1-RPC10), two are shared between Pol I and Pol III (RPAC1, RPAC2) and five are common for all three Pols (RPABC1-RPABC5).[29] Of the RNA Pol III specific subunits, RPC3, 6 and 7 form a subcomplex that is detachable from the rest of the enzyme. It has been shown that these three subunits are not essential for elongation and termination but instead are required for the initiation of the transcription process.[30] Therefore, the finding that the conversion of poly(dA:dT) into poly(rA:rU) is not affected by the absence of RPC3 and RPC7 supports the idea of promoter-independent transcription. For Pol III promoters that contain a TATA-box, the transcription factor TFIIIB binds to DNA by the activity of its TBP subunit.[29] In their study Chiu et al. note that transcription of poly(dA:dT) does not require TBP. Although the authors acknowledge that TBP might still play a role in the in vivo conversion process of poly(dA:dT) templates, TBP did not co-purify with the RNA Pol III complex that was sufficient for in vitro catalysation of RNA Pol III transcription. Thus, this finding further substantiates the idea of promoter-independent transcription. However, Chiu et al. co-purified RNA Pol III subunits using antibodies targeting RPC6 and RPC7 (gene names POLR3F and PPOLR3G). While components of the subcomplex consisting of RPC3, RPC6 and RPC7 might also assemble on poly(dA:dT), their functional activity may not be necessary for the intracellular transcription of poly(dA:dT).

The next question was whether other artificially introduced DNA templates could also drive transcription by RNA Pol III and whether these were also able to exert the immunostimulatory response which was observed for poly(dA:dT). As would have been expected from the observations outlined above, randomly generated dsDNAs were unable to drive type I interferon production. In addition, Chiu et al. report that GCAT or GC sequences were unable to serve as templates for the synthesis of stimulatory RNA species, and the insertion of GCs into a homopolymeric poly(dA:dT) stretches abolished IFN-inducing activity. This suggests that some characteristic feature of poly(dA:dT) is either preferred for transcription by RNA Pol III or that the resulting RNA transcript is ideally "suited" as a ligand for RIG-I. Chiu et al. found that RNA Pol III could not transcribe poly(dG:dC) into the cognate RNA template. However, the question whether other homopolymeric DNA templates are converted into stimulatory RNA species has not been answered so far. Nevertheless, due to its sequence complementarity, poly(rA:rU) forms a stable RNA duplex which renders it a strong ligand for RIG-I.

Immunostimulatory shRNAs are Sensed by RIG-I

On the basis of these findings, we next asked whether RIG-I was also involved in the response to Pol III transcribed interferon-inducing shRNAs. Indeed, we found that the knock-down of RIG-I abrogated the IFNβ promoter activity of an interferon-inducing shRNA construct expressed from a U6 promoter containing plasmid. Similar data were subsequently published by Tang and colleagues.[31] Together these findings underscore the importance of the RNA Pol III—RIG-I—IFN—pathway, not only for promoter-independent transcription but also for RNA transcripts that are generated via the classical promoter-dependent pathway. Our data form a connection between the interferon-inducing activity of shRNAs and their detection by the RNA helicase RIG-I. Importantly, this observation breaks with a previous hypothesis that the delivery of endogenous templates for mediating RNAi would circumvent interferon induction of artificially introduced siRNAs and thus limit non specific effects.[32]

A Role for RNA Pol III in Pathogen Sensing

Finally, the intriguing question remained as to whether the RNA Pol III recognition pathway also operates in a physiological setting to recognize viruses on the basis of their genetic material. Although many DNA viruses hijack the host RNA polymerase system in the nucleus, the majority of their transcripts acquire—just like host genes—a variety of modifications including capping, nucleoside modifications or protection via binding to regulatory proteins. Through these mechanisms, a recognition by RIG-I is prevented. Still, some viruses from the herpes family of DNA viruses have been reported to encode for structural RNAs harboring an unmodified, "free" triphosphate end. This is, for instance, the case for RNA Pol III transcribed EBV-encoded RNA 1 (EBER-1) and EBER-2 derived from Epstein Bar Virus. In contrast to poly(dA:dT), the transcription of the EBER gene locus is accomplished through promoter-directed transcription involving specific RNA Pol III promoters sites thus resembling the mechanism observed for RIG-I stimulatory shRNAs. Furthermore, Chiu et al. report that interferon induction after infection of cells with adenovirus can be abrogated by pretreatment with the Pol III inhibitor ML-60218. While the mechanism is not yet fully understood, it could be that the RNA Pol III encoded VA-I and VA-II transcripts are responsible for this effect.

Interestingly, Chiu et al. also advocate a role for the RNA Pol III-dependent induction of Interferon-β in the immune response to the facultative intracellular, pathogenic bacteria *Legionella pneumophila*. Indirect evidence already exists that, by means of its type IV secretion system, *Legionella pneumophila* injects DNA into the host cell cytoplasm that eventually leads to the induction of type I interferon. While a role for MAVS was postulated in mediating this effect, the same study negated the putative role of RIG-I.[33] Studies in RIG-I deficient cells should help to clarify these discrepancies. In addition, specifying the genomic part that is responsible for the induction of interferon would be interesting. In this regard, another important question is whether this pathway is operational in the cytoplasm, in the nucleus or in both compartments. RNA Pol III is known to be mainly confined to the nucleus, yet there are reports that ascribe RNA Pol III functional activity in the cytoplasm as well. In fact, Chiu and colleagues have immunoprecipitated RNA Pol III subunits from cytosolic lysates, and, in addition, they have shown that RNA Pol III within cytosolic extracts can transcribe poly(dA:dT) into poly(rA:rU). Future studies that will be able to directly investigate the conversion of AT-rich DNA by RNA Pol III are required to clarify this phenomenon (**Fig. 2**).

Conclusion

In summary, these data have uncovered a novel pathway for the recognition of foreign DNA by RNA Pol III. Determining the functional contribution of RNA Pol III for the recognition of other infectious pathogens will be a notable advance in the field of innate immunity, yet it also poses significant technical challenges. One consideration that hampers the straightforward investigation of the RNA Pol III pathway is the existence of additional, but so far uncharacterized, cytoplasmic or nuclear DNA-sensor(s) that could render the RNA Pol III pathway redundant. In addition, current effort is being spent to unravel the precise elements within shRNAs that render them immunostimulatory. Not only will this improve our understanding of endogenous RNAi routes, but this will also be of great benefit for the design of "safe" and highly selective shRNAs.

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