Design and Analysis of Shedding Studies for Virus or Bacteria-Based Gene Therapy and Oncolytic Products

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Guidance for Industry

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Shedding - Gene Therapy

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I. INTRODUCTION

The Center for Biologics Evaluation and Research (CBER)/Office of Cellular, Tissue, and Gene Therapies (OCTGT) is issuing this guidance to provide you, sponsors of virus or bacteria-based gene therapy products (VBGT products)[1] and oncolytic viruses or bacteria (oncolytic products)[2] with recommendations on how to conduct shedding studies during preclinical and clinical development. For purposes of this guidance, the term "shedding" means release of VBGT or oncolytic products from the patient through one or all of the following ways: excreta (feces); secreta (urine, saliva, nasopharyngeal fluids etc.); or through the skin (pustules, sores, wounds). Shedding is distinct from biodistribution because the latter describes how a product is spread within the patient's body from the site of administration while the former describes how it is excreted or released from the patient's body. Shedding raises the possibility of transmission of VBGT or oncolytic products[3] from treated to untreated individuals (e.g., close contacts and health care professionals). This guidance represents FDA's current thinking on how and when shedding data should be collected for VBGT and oncolytic products during preclinical and

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[1] Gene therapy products are all products that mediate their effects by transcription and/or translation of transferred genetic material and/or by integrating into the host genome and that are administered as nucleic acids, viruses, or genetically engineered microorganisms. The products may be used to modify cells in vivo or transferred to cells ex vivo before administration to the recipient. See section III. of FDA's guidance entitled "Guidance for Industry: Gene Therapy Clinical Trials - Observing Subjects for Delayed Adverse Events" dated November 2006. http://www.fda.gov/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/cellularandgenetherapy/ucm072957.htm.

[2] Oncolytic products refer to replication competent viruses or dividing bacteria that are used as therapeutic agents to mediate lysis of tumor cells. Some oncolytic products carry foreign genes (immune modifying genes, genes that enhance oncolysis etc.), and mediate part of their anti-tumor effect by transcription and/or translation of these foreign genes in the host. Hence, oncolytic products that carry foreign genes can also be classified as gene therapy products.

[3] Transmission could occur if the VBGT or oncolytic product is shed in the form of intact viruses or bacteria but not when shed as viral or bacterial degradation products such as nucleic acid fragments.

clinical development and how shedding data can be used to assess the potential for transmission to untreated individuals. This guidance finalizes the draft guidance of the same title dated July 2014.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the FDA's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in FDA's guidances means that something is suggested or recommended, but not required.

II. SCOPE

The products covered by this guidance are VBGT and oncolytic products that OCTGT reviews. The focus of this guidance is shedding studies, including both how and when shedding data should be collected and how shedding data can be used to assess the potential for transmission to untreated individuals.

This guidance does not cover plasmids, peptides, and genetically modified mammalian cells that OCTGT also reviews because, unlike VBGT and oncolytic products, there is no potential for plasmids, peptides, and genetically modified mammalian cells to be infectious or transmissible. This guidance also does not address collection or submission of adverse event information, including those adverse events that could be attributed to shedding. Please see the regulations at Title 21 of the Code of Federal Regulations (CFR) Part 312, specifically 21 CFR 312.32 and 21 CFR Part 600, specifically 21 CFR 600.80, for information on the collection and submission to FDA of adverse event information.

Finally, while assessment of shedding can be utilized to understand the potential risk to the environment, the scope of this guidance does not include shedding as it may relate to potential environmental concerns with respect to a specific VBGT or oncolytic product. For more information on this topic, you may wish to consult FDA's guidance document entitled "Determining the Need for and Content of Environmental Assessments for Gene Therapies, Vectored Vaccines, and Related Recombinant Viral or Microbial Products; Guidance for Industry" dated March 2015.[4]

III. BACKGROUND

VBGT and oncolytic products are derived from infectious viruses or bacteria. In general, these products are not as infectious or as virulent as the parent strain of virus or bacterium because of, in part, the derivation methods and/or modifications made during product development that lead to attenuation. Hence, it is likely that these products are shed to a lesser extent than during

[4] Available at http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm401869.htm.

natural infection by the parent strain. Nonetheless, the possibility that the shed VBGT or oncolytic product may be infectious raises safety concerns related to the risk of transmission to untreated individuals. To understand this risk, shedding studies that are conducted in the target patient population(s) may be appropriate before licensure.

Typically, clinical shedding studies are not stand-alone studies but are integrated into the design of a safety or efficacy trial. Because there are many product-specific factors and patient-specific factors that can influence the design of a shedding study, sponsors should consult with OCTGT in the early stages of product development for specific recommendations as to their product.

IV. WHY COLLECT SHEDDING DATA DURING PRODUCT DEVELOPMENT?

Shedding studies should be conducted for each VBGT or oncolytic product to provide information about the likelihood of transmission to untreated individuals because historical data alone may not be predictive of the shedding profile. Shedding data can be used to evaluate measures to prevent transmission. Shedding data collected during product development should provide a clear and comprehensive understanding of the shedding profile of VBGT or oncolytic products in the target patient population(s). Note that it may be appropriate to describe these data in the package insert for an approved Biologics License Application (BLA).

To inform the design of human shedding studies, shedding data may be collected in animals following administration of the VBGT or oncolytic product. These data can help estimate the likelihood and potential shedding profile in humans, particularly when there is concern about transmission to untreated individuals. However, such data cannot substitute for human shedding studies for several reasons. For example, a VBGT or oncolytic product may be derived from a human-specific strain; therefore, animals may not adequately predict the shedding profile in humans. Similarly, various animal species/models may not adequately address patient-specific factors, such as differences in the immune status at the time of product administration, which may contribute to the potential for shedding in humans (for more details refer to section VII.B. of this guidance).

Product-specific variables may also affect shedding. For example, the biological characteristics and route of administration (entry) of VBGT or oncolytic products can be different from that of the parent strain of viruses and bacteria. Specifically, these products may be:

- Derived from laboratory-adapted wild-type, attenuated or engineered strains that may not have been characterized in humans in prior studies.

- Replication competent or incompetent viruses; viruses that can infect a host cell and amplify to produce progeny are replication competent and those that can infect a host cell but cannot establish an infection, amplify, and produce progeny are replication incompetent.

- Dividing and/or auxotrophic bacteria; auxotrophic bacteria are unable to synthesize an organic molecule required for their growth and division but when this molecule is available with the other nutrients they require, growth and division of the bacteria may occur.

- Introduced into the human body through unnatural routes and hence, the infectivity, replication, persistence and shedding from the human body may be different than that of the parental strains.

- Engineered to carry transgenes, e.g., tropism-altering genes, immune modifying gene(s) or genes that enhance oncolysis.

V. COLLECTION OF SHEDDING DATA IN PRECLINICAL STUDIES

The decision to assess shedding in preclinical studies[5] is based on the biological characteristics, derivation, and genetic make-up of the VBGT or oncolytic product. For example, preclinical shedding data may be requested for an oncolytic or a replication competent VBGT product, if:

- Humans have not been previously exposed to the product, as in the case of a non-human bacterial or viral strain.

- The product has been administered to humans, but has been modified to achieve a different in vivo tropism than the parent strain.

- The product has been previously administered to humans; however, a change in the route of administration is proposed.

- Humans have not been previously exposed to the product, and the route of administration differs from the natural route of exposure/infection.

The use of the animal species/model(s) is an important factor that can affect the biological relevance of the shedding profile generated in the animal. Considerations include the permissiveness or susceptibility of the animal to infection from the VBGT or oncolytic product under investigation, and any preexisting immunity that may affect infectivity or product clearance.

Collection of shedding data is an endpoint that can be included in preclinical studies designed to collect other data, such as safety and biodistribution. The decision to include an assessment of the shedding profile of a VBGT or oncolytic product in an animal study will depend on various

[5] For general information about preclinical assessment, including study design, of these products see FDA's guidance entitled "Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products" dated November 2013, http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm376136.htm.

product-specific factors, as described above and in sections I. and IV. of this guidance. We recommend that sponsors initiate communication with the Pharmacology/Toxicology staff in OCTGT early in their product development program to discuss the need for generating a shedding profile for their VBGT or oncolytic product in animals, and the planned methodology to collect this shedding data.

VI. DESIGN OF SHEDDING STUDIES: GUIDING PRINCIPLES

The key aspects in the design of shedding studies are: the choice of clinical samples that are collected from subjects in a trial (e.g., feces, urine, nasal swabs); the frequency of sample collection and duration of the monitoring period; and the assay methodology selected to test for the presence of the shed VBGT or oncolytic product in the clinical sample (Ref. 1).

To guide the design of shedding studies, the following should be considered:

A. Biological Characteristics

- Replication competence: The ability of the VBGT or oncolytic product to multiply and amplify in the human host greatly affects how it is disseminated in the body and may increase the extent and duration of shedding.

- Immunogenicity: When the VBGT or oncolytic product is derived from viruses or bacteria that elicit a strong immune response, the product may be more rapidly cleared from circulation than a poorly immunogenic product, and may be shed for a shorter duration. Similarly, when a product is administered multiple times, the product may be shed for a shorter duration in the later dose cycles than after the immune-priming first or early doses.

- Persistence and latency: The duration of a shedding study may be longer if the VBGT or oncolytic product exhibits persistence or latency-reactivation in the host, as in the case of an oncolytic herpes virus product that is capable of latency (period of time during which a virus is present in the host without producing overt clinical symptoms). Shedding of such products may be intermittent and unpredictable.

- Tropism: Tropism of the product may affect what samples should be collected to assess shedding. For example, VBGT or oncolytic products that are engineered to carry tropism modifying gene(s) or mutation(s) may exhibit an altered shedding profile than the parent virus because of retargeting of the product to different tissues or organs.

- Stability of product attenuation: It is common for VBGT or oncolytic products to be attenuated either for replication in normal (non-tumor) cells, or for loss of virulence or latency in the human host. However, for some products that have a higher potential for recombination or reversion in the patient, the shedding pattern and/or what is shed may change.

B. Route of Administration

In addition to the tropism of the VBGT or oncolytic product, the route of product administration should be considered in the selection of sample types to collect in a shedding study. For example, to assess shedding in patients administered an oncolytic virus by the intradermal route, we recommend the collection of skin swabs at the site of injection, in addition to the other samples routinely assessed for shedding (e.g., urine, feces, and saliva). Similarly, we recommend the collection of nasopharyngeal washes when an oncolytic virus is administered by inhalation or via the intranasal route.

VII. COLLECTION OF SHEDDING DATA IN CLINICAL STUDIES

Shedding data collected in clinical studies provides a shedding profile of a product in the target patient population and is used to estimate the potential of transmission to untreated individuals. Depending on the shedding profile, it may be appropriate to include the information on shedding in the Investigator Brochure and in the Informed Consent for Investigational New Drug (IND) studies. Depending on the shedding profile, it also may be appropriate to include shedding data in the package insert for licensed products. This information will inform patients and physicians if shedding could occur with the use of a VBGT or oncolytic product, the potential for transmission of the product to untreated individuals, and of the measures to take to prevent such transmission.

A. When to Collect Shedding Data in Clinical Studies?

- For VBGT and oncolytic products classified as replication competent, we recommend that sponsors begin collecting shedding data in Phase 1 trials. Considering that replication competent products are associated with a higher potential for release as infectious viruses or bacteria, sponsors may need to continue collecting shedding data during Phase 2 and Phase 3, after a dose and regimen have been determined, to better characterize shedding.

- For VBGT products that are classified either as replication incompetent or replication deficient, we recommend that sponsors collect shedding data later in product development (e.g., during Phase 2 studies), after a dose and regimen have been determined. Compared to shedding of replication competent products, shedding of replication incompetent or replication deficient products is expected to be low, for a limited duration, and associated with a lower potential for release as infectious viruses or bacteria.

- After shedding data are collected in early phase studies, it is not uncommon that dose, route, regimen (frequency of product administration, concomitant therapy, preconditioning regimens, etc.), or indication are modified. These changes can alter how product is shed. In such cases, shedding data collected in early clinical trials may not be adequate or relevant to predict the shedding profile of the product in its current state. Additional data should be collected in subsequent clinical trials where the route, dose regimen, and indication are the same as in the pivotal trial.

B. Study Design

The plan to collect shedding data in clinical studies can be based on prior clinical experience with the same or similar product, but when there is no such experience, as in the case of first-in-human VBGT or oncolytic products, the shedding profile generated in animals can be informative. We recommend that sponsors prospectively design and incorporate the sampling plan in the clinical study to collect shedding data.

There are four critical choices in the design of a sampling plan:

- Frequency of sample collection;
- Duration of sample collection;
- Type(s) of samples collected; and
- Storage conditions for types of samples collected.

However, there are many aspects that can influence these choices, as described earlier in section VI. of this guidance and further elaborated below.

- Frequency of sample collection: Shedding is most likely to occur in the period immediately following product administration, irrespective of replication competence of the VBGT or oncolytic product. A second peak of shedding may be noted in the days/weeks after administration of a replication competent product as a result of its multiplication/amplification in vivo. Accordingly, sampling should start immediately after product administration, with frequent sampling during the initial weeks following treatment to capture the shedding pattern accurately (e.g., sampling on day 1, 3, 7, 10 and then weekly). Analysis of samples should continue until three consecutive data points are obtained at or below the limit of detection (LOD) of the shedding assay. If the level of shedding does not reach the LOD of the assay but there is a continual decreasing trend, collection should continue until the results demonstrate that a plateau has been reached in at least three consecutive data points.

- Duration of sample collection (monitoring period for shedding):
  - In general, when the VBGT or oncolytic product is a replication competent virus or bacteria, the monitoring period for shedding is longer (for sample collection) as compared to when the product is replication incompetent or replication deficient. This is because you will want to capture the second peak of shedding associated with multiplication or amplification in vivo.
  - The immune status of the patient population should be considered. Patients who are immune compromised may have an extended or even different shedding profile than their immune competent counterparts. For replication competent products, the immune competence of the patient population is a relevant factor because many of these products are used in cancer patients who have had immunosuppressive chemotherapy. When treated with replication competent VBGT or oncolytic products, immunosuppressed patients may become persistently infected and may shed the product for extended periods of time (Ref. 2). Therefore, the monitoring period for shedding may be longer for immunosuppressed patients treated with a replication competent VBGT or oncolytic product.
  - When a VBGT or oncolytic product is administered in multiple cycles, or when there is pre-existing immunity, the duration of shedding may be shortened because of product-specific immune responses. Data from single dose administration of product may be used to guide the timing of sample collection following multiple administrations.
  - If an oncolytic product is based on a virus that has the potential for latency reactivation, we recommend the collection of additional samples for shedding analysis when clinical signs warrant, i.e., when patients show signs of infection due to reactivation.
- Type(s) of samples collected: The types of clinical samples (e.g., urine, fecal swabs, saliva, etc.) collected to assess shedding depend on a variety of factors including the route of administration of the product, the tropism of the virus or bacteria, the natural route of transmission and shedding of the parent virus or bacterium from which the product is derived (as described in section VI. of this guidance), and biodistribution or shedding data from preclinical studies. For example, if an oncolytic herpes virus product is administered intradermally for treatment of skin cancers, there is the potential of transmission of the oncolytic herpes virus product through infected scabs/skin secretions because that is a natural route of transmission for herpes viruses. In this case, skin swabs or dressing from injection sites should be analyzed for shedding. Likewise, if an oncolytic adenovirus product is administered intranasally, there is the possibility of transmission of the adenovirus product through respiratory secretions, therefore, nasopharyngeal swabs or washes should be collected in the shedding study. For tropism-modified products, knowledge about the route of transmission

and shedding of the parent virus or bacterium may not be relevant or sufficient to guide in sample collection.

- Blood is not typically analyzed for shedding but should be collected as part of pharmacokinetic analysis to understand the extent of product dissemination from the site of administration and the rapidity of product clearance. This information can be particularly useful to assess the extent of product shedding when limited vascular spread is expected for products administered locally (e.g., intratumoral, intramuscular, intracranial, subretinal routes).

- Storage conditions for type(s) of samples collected: The appropriate storage conditions for different type(s) of samples need to be established in order to minimize degradation of product-specific nucleic acids or loss of product-specific infectivity or bacterial viability. Multiple aliquots of samples may be needed for different tests, such as for a product-specific infectivity assay after a sample is scored positive for product-specific nucleic acids.

VIII. ANALYTICAL ASSAYS TO MEASURE SHEDDING

An analytical assay that measures shedding is designed to detect product in the clinical sample, either by detection of nucleic acids or for the presence of infectious viral particles or dividing bacteria. Based on the design and output (nature of the assay readout), shedding assays can vary greatly in their performance and suitability. Hence, the choice of a shedding assay can greatly affect the quality of the data collected, and is important in the generation of meaningful shedding data, i.e., data that provides a complete shedding profile for a product and can be used to estimate the potential for transmission to untreated individuals.

We recommend that sponsors consider the following in the selection of the analytical assay to measure shedding:

- At least one of the assays used to measure shedding should be quantitative. We recommend that sponsors report the extent of shedding of VBGT or oncolytic products in terms of the number of genome copies or infectious units to provide a quantitative assessment of shedding. Often an assay with a quantitative readout, like quantitative polymerase chain reaction (qPCR), is used because of the ease of performing/standardizing the assay, high throughput format, rapid turnaround time, and assay sensitivity.

- Because detection of nucleic acids by qPCR may not indicate the presence of infectious virus, for replication competent products, detection of nucleic acids should be followed up with infectivity or growth-based assays. Replication competent products are capable of growth or multiplication in humans and if shed, can be infectious. Since only infectious viruses or bacteria are potentially transmissible, we recommend that sponsors follow a step-wise approach for the analysis of shedding of replication competent products. Specifically, a steady rise in the PCR signal for

product-specific nucleic acids in clinical samples collected over successive time-points is suggestive of bacterial growth or viral multiplication in vivo. In such cases, clinical samples should be further analyzed for infectivity in cell culture (for viruses) or growth (for bacteria).

- If shedding of conditionally replicating VBGT or oncolytic products is noted by qPCR assay at a level above the LOD, we recommend that sponsors further characterize the shed material to confirm infectivity or growth because such products could be shed in their infectious form even if replication is confined, mostly, to tumors or to a particular tissue-type. The assessment of infectivity for conditionally replication competent products, such as conditionally replicating adenovirus or auxotrophic bacteria, should take into account product-specific in vitro cell culture or growth conditions; for example growth in differential media for auxotrophic bacteria followed by a selective method for product identification.

- There are many different approaches to assess infectivity or growth with assays that have a quantitative read-out (Ref. 3). For example:

    - For detection of infectious viruses: Assays that measure infectivity in terms of Tissue Culture Infectious Dose 50 ($TCID_{50}$), plaque-forming units (PFU), focus-forming units (FFU).

    - For detection of dividing bacteria: Assays that measure bacterial growth in colony forming units (CFU).

  Sponsors may justify limiting the shedding analysis of a replication competent or conditionally replicating product to qPCR assay, if:

    - A correlation between qPCR and the infectivity assay or growth-based assay is established, and the signal noted in the qPCR assay is at or below the LOD of the infectivity or growth-based assay; or

    - The cell culture step in an infectivity assay is demonstrated to be unsuitable for the analysis of clinical samples with complex composition such as excreta due to adverse effects on cell viability.

- Shedding analysis of replication incompetent or deficient products by qPCR may be adequate. Most VBGT products are replication incompetent or replication deficient; for example, adeno-associated virus (AAV) vectors, E1-deleted adenovirus (Ad) vectors, and some herpes virus vectors (HSV). Replication incompetent or replication deficient products are incapable of multiplying in humans, and therefore, are shed to a lower extent and in a form that is incapable of establishing an infection. Hence, qPCR may be adequate as the primary assay to assess shedding of replication incompetent or replication deficient products.

- Use of a qualitative assay to assess shedding may be justified. When a qualitative shedding assay is demonstrated to be more sensitive than a quantitative assay in the detection of a specific product, a sponsor may justify the use of the former. However, if there is detectable shedding with a qualitative assay, a follow-up quantitative analysis should be performed to understand the extent of shedding in positively scored samples.

- Shedding assay(s) should be demonstrated to be specific, sensitive, reproducible and accurate. We recommend testing of clinical samples in a shedding assay in replicates to determine reproducibility. The specificity of the assay should be well understood to avoid false-positive or false-negative results, particularly since retesting is not always feasible with clinical samples that are limited in quantity, such as nasal or skin swabs. The sensitivity of the assay should be determined in terms LOD and the limit of quantitation (LOQ), if using a quantitative assay. While the Agency does not expect shedding assays to be validated, the assays should be qualified to meet minimal performance capabilities and be suitable for the intended purpose.

The effect of sample type and composition on assay performance should be well understood. Clinical samples such as feces and urine are rich in complex organic matter that can adversely affect the performance of an assay and lead to an underestimation of shedding. Also, samples such as feces, saliva, and nasal swab are rich not only in host proteins and nucleic acids but also in the body's natural flora and in circulating strains of viruses and bacteria from the environment. Thus, the assay conditions should be optimized to selectively analyze for the product under investigation. For that, the specificity of the reagents used in the assay should be assessed and the quality of the reagents should be controlled. Certified and contaminant-free reagents should be used in the analysis of clinical samples in a shedding assay.

Interference from clinical sample matrix can lead to a false-negative result or an underestimation of the amount of shedding. For example, clinical samples like urine, saliva, and feces are rich in proteases, nucleases, ions and salts that can affect the amplification process in a PCR; specifically, nucleases in saliva/feces can degrade template DNA, bile salts in feces or urea in urine can affect the activity of thermostable DNA polymerases in the PCR mixture. When PCR inhibition is suspected due to interference from components in a clinical sample, the clinical sample can be diluted to a limited extent to reduce the interfering component. Since dilution of the clinical sample also leads to template dilution, the sensitivity of PCR assay should be assessed in each assay run. For that, each diluted sample should be tested in parallel with one that is spiked with a reference standard or positive internal control prior to dilution. If interference cannot be decreased by limited sample dilution, alternative or additional extraction procedures should be considered to remove the interfering component(s) in the clinical sample.

An underestimate of the level of shedding may also result due to degradation of viral or bacterial nucleic acids in enzyme-rich clinical samples such as feces and saliva during storage, handling/shipping and nucleic acid extraction. To account for such effects, we recommend that mock/donor sample types be spiked, soon after collection, with the reference standard or internal

positive control and the percent recovery of the reference standard or internal positive control should be determined on a one-time basis. Sample collection, storage, shipping, extraction and analysis should be performed with the same methodology as that planned for the clinical (test) samples.

IX. ANALYSIS OF SHEDDING DATA

In order to assess the potential of transmission to untreated individuals due to shedding, the analysis of shedding data for VBGT or oncolytic products should address the following:

A. The Nature of the Shed Material

When clinical samples are scored positive for product in a shedding assay, the subsequent analysis of these samples should provide answers to the following questions:

1. Do the clinical samples contain product-specific nucleic acids (full-length genomes) suggestive of the presence of infectious viruses or bacteria or do the clinical samples contain mostly degraded product-specific nucleic acid (genome) fragments found in the absence of infectious viruses or bacteria?

An example of a clinical sample containing product-specific nucleic acid (full-length/complete genomes) suggestive of the presence of infectious viruses is one in which product-specific nucleic acids are amplified (by PCR) after treatment with nucleases. Under such conditions, the only genomes that are amplifiable are the full-length/complete genomes protected within intact nuclease-resistant viral particles that may be infectious. When nuclease treatment is not feasible, amplification of full length/complete viral genome by long PCR may suggest the presence of infectious viral particles. If only small product-specific nucleic acid fragments are amplifiable, then the clinical sample is not likely to contain infectious viruses.

2. Can viral or bacterial growth attributable to the shed product be detected in the clinical sample?

Ideally, the shedding assay should be able to discern infectious from non-infectious viruses, or dividing from non-dividing bacteria. We refer you to section VIII. of this guidance for details on shedding assays and for our recommendations. If qPCR is the only assay you have relied on in your shedding analysis of replication competent products, or the shedding assay screens for small genome fragments of the product, then we will assume that the shed material in the positively scored samples is infectious.

B. The Extent of Shedding

In the analysis of shedding data, the extent of shedding noted for each sample type as a factor of time, dose (amount of product administered) and regimen (number of doses) should be reported for all the patients monitored in the study. Raw data in the shedding report should be accompanied by a corresponding analysis that is comprehensive and describes the following:

1. The number of patients that are shedding as a percentage of the total patients in the study for each sample type, dose and regimen studied.

2. The duration of shedding, including the first and last day of shedding, and the peak period(s) of shedding in each sample type. The period when shedding stops in most patients in the study also should be clearly identified.

3. The clinical sample(s) where shedding was consistently noted (type and time point) and samples that were consistently negative for all the patients in the study.

4. The quantity of product shed in a clinical sample. The amount shed should be reported taking into account the final volume/mass of the clinical sample, (e.g., 10 PFU of virus per mL of urine, or 10 CFU of bacteria per mL of urine, or 10 genome copies per microgram of stool). When assessing the quantity of shedding, you should factor in the stability of the product in the clinical sample, and whether there could be an underestimate of the level of shedding because of loss during sample storage, handling and shipping (for details, please refer to section VIII. of this guidance).

Note that your analysis of shedding data should be accompanied by a summary of the shedding profile of the product in patients treated for a specific indication. While it is common practice in clinical development of VBGT and oncolytic products to study a product for different indications in multiple trials, the shedding pattern may be distinct in each study population. We recommend against pooling shedding data from multiple trials in which the same product is studied for different indications because results from a shedding study in a given indication may not be generalizable to other indications.

Finally, we recommend that the shedding data be submitted in a format as described in the next section.

X. WHAT TO INCLUDE IN A CLINICAL SHEDDING STUDY REPORT

In order to address the potential for transmission to untreated individuals due to shedding of VBGT or oncolytic products from the patient, a full shedding report should be provided in the BLA.[6] The following should be provided in the report:

[6] Interim data and study reports can be submitted in your IND annual report, for FDA review, if guidance is desired.

A. A comprehensive shedding profile of the product in the target patient population(s) which includes the following:

1. Background information on the product: Derivation history; biological characteristics of parent viral or bacterial strain; route of transmission of parent strain; replication competence; attenuation; and tropism of the product.

2. A summary of the biodistribution profile in animal models and the findings from preclinical shedding studies, if conducted.

3. The rationale for both the clinical shedding study design (i.e., choice of clinical samples, frequency and procedures of collection and storage), and the analytical method selected to assess shedding.

4. Your data collection/sampling plan and your procedures for storage, shipping, and handling of the product.

5. An assay description that includes the following:

    (a) Test sample preparation or nucleic acid extraction procedures including the dilution factor and amount of nucleic acid extracted per sample.

    (b) If you use qPCR assays, provide for each assay the sample volume, amount of nucleic acid per reaction, cycle numbers, primers, and size of the amplified DNA.

    (c) If you use infectivity/growth-based assays, provide the permissive cell line/growth media, the conditions for adsorption and infection or growth, and the nature of the read-out (TCID50, FFU, PFU or CFU; or Cycle threshold (Ct) for assays with qPCR read-out).

    (d) Assay qualification, controls and sensitivity: Description of the qualification studies, standards, spikes, controls, number of replicates, assay variability and sensitivity (LOD and LOQ, if applicable).

6. Analysis of shedding data:

    (a) Tabulation and/or graphical representation of the shedding data.

    (b) Analysis of the data and summary of the findings from the study.

7. Your estimate of the potential for transmission to untreated individuals of the product.

In this guidance, see section VIII. for a discussion of analytical assay types and conditions, section VII.B. for a discussion of clinical sample collection, section IX. for

analysis of shedding data, and section XI. for details on assessing the potential for transmission to untreated individuals due to shedding.

B. As needed, data and analysis of clinical monitoring for transmission to untreated individuals in the target patient population (refer to section XI.B. of this guidance for additional information).

C. Other relevant information on the ability of the VBGT or oncolytic product, or its parental/related strain of viruses or bacteria to potentially infect humans and cause disease. When there is the potential to cause disease in humans, the following should be discussed:

1. The spectrum of disease symptoms caused by the parental strain, including atypical presentation of the disease, or occurrence of asymptomatic shedding;

2. The attenuation of the product compared to the parent strain of the virus or bacterium circulating in the community;

3. The natural or acquired immunity of the general population that could potentially protect against infection from the shed product;

4. The therapeutic options to treat the infection/disease in case of transmission to untreated individuals of the shed product; and

5. Preventive/containment measures that can limit spread of the shed product beyond the treated individual to minimize exposure of third parties, particularly, immune-compromised adults, neonates and seniors. Note that the data you collect on onset and duration of shedding can inform appropriate preventive/containment measures. For example, if peak shedding occurs soon after treatment when the patient is monitored in a health-care setting, then the possibility of transmission is mainly confined to health care professionals (HCP) and individuals that come into close contact with the patient. If shedding is prolonged or if there is a second peak of shedding in the days following discharge of a patient from a health care setting, there is the possibility of transmission to contacts beyond the health care and home setting.

XI. ASSESSING THE POTENTIAL FOR TRANSMISSION TO UNTREATED INDIVIDUALS DUE TO SHEDDING

Our current understanding is that in most cases, the potential for transmission to untreated individuals is extremely low when VBGT or oncolytic products are shed because of the derivation methods and/or modifications that are designed to attenuate the product when compared to the parent strain of virus or bacterium. Nevertheless, you should discuss the potential for transmission based on the analysis of the shedding data collected in the clinical studies and taking into consideration the factors described below.

A. What Information in the Shedding Data Can be Used to Assess Potential for Transmission to Untreated Individuals?

- Whether the VBGT or oncolytic product was shed.
- Whether the shed product was determined to be infectious.
- Whether the amount of infectivity in the clinical samples was comparable to that needed to initiate infection in a third party. For example, adenovirus infective dose is reportedly >150 PFU when given intra-nasally (Ref. 4) or lower when aerosolized (Ref. 5). The minimum infectious human dose may vary when viruses or bacteria are administered or acquired through different routes, or among different strains, but for many disease-causing viruses and bacteria, the minimum infectious dose in humans may be undefined.
- Whether the clinical sample containing the shed product represents the natural route of transmission. For example, a respiratory virus that is shed in feces may not be as infectious and transmissible when compared to that shed in nasopharyngeal secretions.

B. Monitoring Untreated Individuals for Transmission

Because transmission to untreated individuals is an extremely low probability event, monitoring such individuals for transmission is usually not required during the clinical development of a product. However, if there is a potential for transmission, additional data will be needed to assess that possibility; in which case, we recommend that sponsors consult with OCTGT in connection with developing a monitoring plan.

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OPINION/HYPOTHESIS
Molecular Biology and Physiology

RNAs That Behave Like Prions

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ABSTRACT The term "prion" was originally coined to describe the proteinaceous infectious agents involved in mammalian neurological disorders. More recently, a prion has been defined as a nonchromosomal, protein-based genetic element that is capable of converting the copies of its own benign variant into the prion form, with the new phenotypic effects that can be transmitted through the cytoplasm. Some prions are toxic to the cell, are able to aggregate and/or form amyloid structures, and may be infectious in the wild, but none of those traits are seen as an integral property of all prions. We propose that the definition of prion should be expanded, to include the inducible transmissible entities undergoing autocatalytic conversion and consisting of RNA rather than protein. We show that when seen in this framework, some naturally occurring RNAs, including ribozymes, riboswitches, viroids, viroid-like retroelements, and PIWI-interacting RNAs (piRNAs), possess several of the characteristic properties of prions.

KEYWORDS piRNA, prions, ribozymes, viroids

Mammalian prions: a classical definition. The studies of a peculiar class of infectious diseases in mammals, i.e., transmissible spongiform encephalopathies (TSEs), and the hunt for the elusive agent that causes these diseases, resulted in the notion of an infectious protein, or "prion." The known prion-caused TSEs include kuru, Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, and fatal familial insomnia in humans, as well as scrapie, bovine spongiform encephalopathy, chronic wasting disease, and several other related diseases in mammals. The early evidence in favor of the protein-only agent for scrapie was based on the observations that the infectious subcellular fraction was highly resistant to UV irradiation, unlike the nucleic acids (summarized in reference 1), that the protocol for purification of the infectious moiety required the conditions used to purify certain proteins rather than RNA, and that the estimated molecular weight and other properties of the main component of such a fraction were close to that of a modest-size protein rather than, for example, a virus nucleic acid (2, 3). This was backed up with experiments designed to exclude a role of small infectious RNAs, in particular viroids, in the etiology of scrapie (4, 5). As early as in 1967, John S. Griffith outlined several possible mechanisms by which a protein could become inherited as a nonchromosomal genetic element. One of those hypothetical mechanisms stated that a prion is a modified form of a cognate cellular protein, which may bind to the normal form of the same protein (in the simplest case, forming a heterodimer of one prion and one normal copy of the protein) and then turn the normal form into another copy of the prion (6). This predicted mechanism was borne out by the evidence and is at the core of the current definition of any prion.

In mice, which are susceptible to the sheep scrapie disease, the *Sinc* gene controlling the incubation period of the disease was identified in 1968 (7). In 1982, it was shown that *Sinc* encodes protein PrP, which copurifies with the prion fraction (2). Animals lacking the PrP-encoding gene are generally not susceptible to the TSE agents, have a

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normal life span, do not show abnormalities in the neural system, and do not propagate the prion. In contrast, when animals that encode the wild-type PrP are infected with the TSE agent, they accumulate protease-resistant aggregates of PrP in neural tissues. These aggregates consist of the prion form of PrP, designated PrP^{Sc}, which has a high percentage of residues within β-sheets and a large protease-resistant core compared to the normal soluble form PrP^{C}. The β-sheet-rich amyloid aggregates of PrP^{Sc} can be obtained *in vitro* from purified recombinant derivatives of PrP^{C}. Indeed, a line of mice has been developed that, when injected with these aggregates, shows the signs of the disease, and their brain extracts are infectious to many mice lines (8), finally concluding Koch's triad (see reference 9).

Thus, the canonical definition of prion may include the following: a prion is a protein that is encoded by the cell but is either benign or possibly useful to the organism, hence the preservation of its gene in the genome. Infrequently, it may be transformed into a disease-causing or toxic prion form; the complete set of factors that cause such a transformation is not known but may include physiological stress. Prion causes the conversion of other, benign forms of the same protein into the prion form, i.e., it propagates within the organism, and it is also transmissible to other organisms or sometimes to a different species. The known mammalian prions form amyloids and cause neural phenotypes (Table 1). Notably, the whole infectious cycle does not require any template-directed amplification of a nucleic acid, other than the synthesis of the mRNA that is translated into the benign form of a protein.

A broadened definition: prions in fungi. Many extrachromosomal genetic elements, including nucleic acid-based viruses and plasmids, are known in the budding yeast *Saccharomyces cerevisiae*. Another subset of yeast cytoplasmic inherited factors have been shown to resemble prions in several ways. In one of the first examples, the extrachromosomal element [URE3], which manifests itself by interfering with nitrogen catabolite repression, cannot propagate in the yeast mutant strains lacking the chromosomal copy of the *URE2* gene (10). The product of *URE2*, a glutathione peroxidase-like protein, Ure2p, is able to bind to two transcription factors and thereby repress the genes encoding the subsystem for the utilization of suboptimal nitrogen sources whenever nitrogen is in abundant supply. The phenotype of the *ure2* mutants, i.e., the derepression of the systems for utilization of poor nitrogen sources, is the same as the phenotype of [URE3], even though there is no [URE3] in *ure2* cells. This led Reed B. Wickner to the idea that [URE3] is a prion of the Ure2p protein—the form of Ure2p in which the "normal" repressor function of the protein is disabled, but the ability of [URE3] to convert Ure2p into extra copies of [URE3] is activated (11). Indeed, [URE3] has been shown to consist of the conformationally altered Ure2p, which can convert "normal" copies of Ure2p into more of [URE3] and form amyloid (12, 13).

In the last 25 years, many other proteins with prion-like behavior have been identified in yeast and other fungi; two of the best-studied ones are [PSI] of *S. cerevisiae*, the prion form of Sup35 protein that is one of the two subunits of the translation termination complex Sup35-Sup45 (14), and [Het-s] of *Podospora anserina*, the prion form of the HET-s protein involved in heterokaryon incompatibility, a cell death reaction preventing mating of genetically distant strains and likely protecting the cells from infection by exotic viruses and plasmids—an example of a beneficial prion (15). The molecular mechanisms of the prion-like behavior of those proteins *in vivo* and *in vitro* have been studied in some detail, and the debates on the possible biological role of prions, most of which seem to be disadvantageous to their hosts, are ongoing (16–21).

With the extension of the concept of prion to the proteins conferring inheritable phenotypes to yeast and other fungi, the prion definition was modified (Table 1 and Fig. 1). The neural diseases are not applicable to yeast, and even the disease/sickness/loss of fitness in the host is not universal in yeast prions—in the expanded definition, these are replaced by a screenable phenotype. Other integral components of the prion definition, however, still hold true for the yeast prions. Specifically, prion proteins are encoded by cellular genes; the benign form of a protein may be converted into a prion

TABLE 1 Criteria and definitions for prions

Classical criterion of a prion in mammals	Expanded prion definition: fungi and other organisms	RNA prion definition in this work	Agreement with the RNA prion definition by genetic element(s):			
			Badelt-Flamm-Hofacker construct	Ribozymes, riboswitches	Viroids	piRNA produced by the ping-pong mechanism
Disease-causing protein	Protein causing a specific phenotype	RNA is associated with a specific phenotype not due to its encoded protein	Not specified	Yes	Disease- or phenotype-causing RNA	Yes
Disease is inducible	Phenotype is inducible	Phenotype is inducible	Conformation switch is inducible	Yes, e.g., by metabolites	Can be engineered	Yes
Disease is transmissible	Phenotype/condition is transmissible	Phenotype/condition is transmissible	Not specified	Yes, by cell division	Yes	Yes, to the progeny
Causative protein is encoded chromosomally, but prion is inherited extrachromosomally	Yes	Yes; RNA prion is encoded chromosomally but inherited extrachromosomally	Not applicable	Yes	Yes	Yes
Benign and prion forms are the same at the sequence level	Protein processing is allowed	RNA processing is allowed	Yes	Yes. *glmS* ribozyme-riboswitch self-cleaves to activate gene expression	Yes (the benign form is a concatemer of the infectious form)[f]	siRNA[g] precursors overlap but are not identical; siRNAs also overlap but are not identical
Rare conversion from benign to prion form	Yes	Rare conversion of RNA from benign to prion form	Yes	Yes; *glmS* transcript stays inactive until GlcN6P concentration rises	Can be engineered	Yes; precursor transcripts do not suppress TE transcription, but piRNA does
Prion form converts a benign form of the same protein to the prion form[a]	Yes	Prion RNA converts a benign form of the same RNA to the prion form	Yes	*glmS* has been engineered to cleave in *trans*	Condition is reversed; "benign" multimers self-cleave into the monomeric pathogenic form[f]	Primary siRNAs induce production of secondary siRNAs, which enable the full extent of silencing
More induction under stress condition	Not universal?[b]	Maybe	Not applicable	Can be engineered	Can be engineered	Yes
Transition to prion state increases the proportion of parallel beta strands and the rate of amyloid formation	No[b]	Not applicable	Not applicable	Not applicable	Not applicable	Not applicable

(Continued on next page)

TABLE 1 (Continued)

Classical criterion of a prion in mammals	Expanded prion definition: fungi and other organisms	RNA prion definition in this work	Agreement with the RNA prion definition by genetic element(s):			
			Badelt-Flamm-Hofacker construct	Ribozymes, riboswitches	Viroids	piRNA produced by the ping-pong mechanism
Neural phenotype	No[c]	No	No	Possible, but not related to TSE	Not applicable in plants, not known in animals	Possible, but not related to TSE
	Yeast: prions are reversibly curable[d]	Yes[d]	Not applicable	Maybe[d]	Maybe	Yes[d]
	Yeast: overexpression of the gene increases the rate of prion emergence	Yes	Not applicable	Maybe	Maybe	TE overexpression is predicted to induce the ping-pong system
	Yeast: phenotype mimics loss of function of the gene[e]	Maybe	Not applicable	Maybe	Maybe	Gain of function

[a]This property may be observed *in vivo* and *in vitro*; other prion properties depend on cell (mal)function for full expression.
[b]Not in yeast ProB.
[c]Not applicable to any of the yeast prions.
[d]Distinguishes prions from other infections agents (viruses, plasmids) but does not distinguish prions from the cases of normal gene regulation.
[e]This is true only if the prion form is inactive; if prion is active, then its phenotype is similar to gain of function of the encoding gene.
[f]Distinction between benign and pathogenic form may be less clear if the mechanism of action of viroids is through small RNA.
[g]siRNA, small interfering RNA.

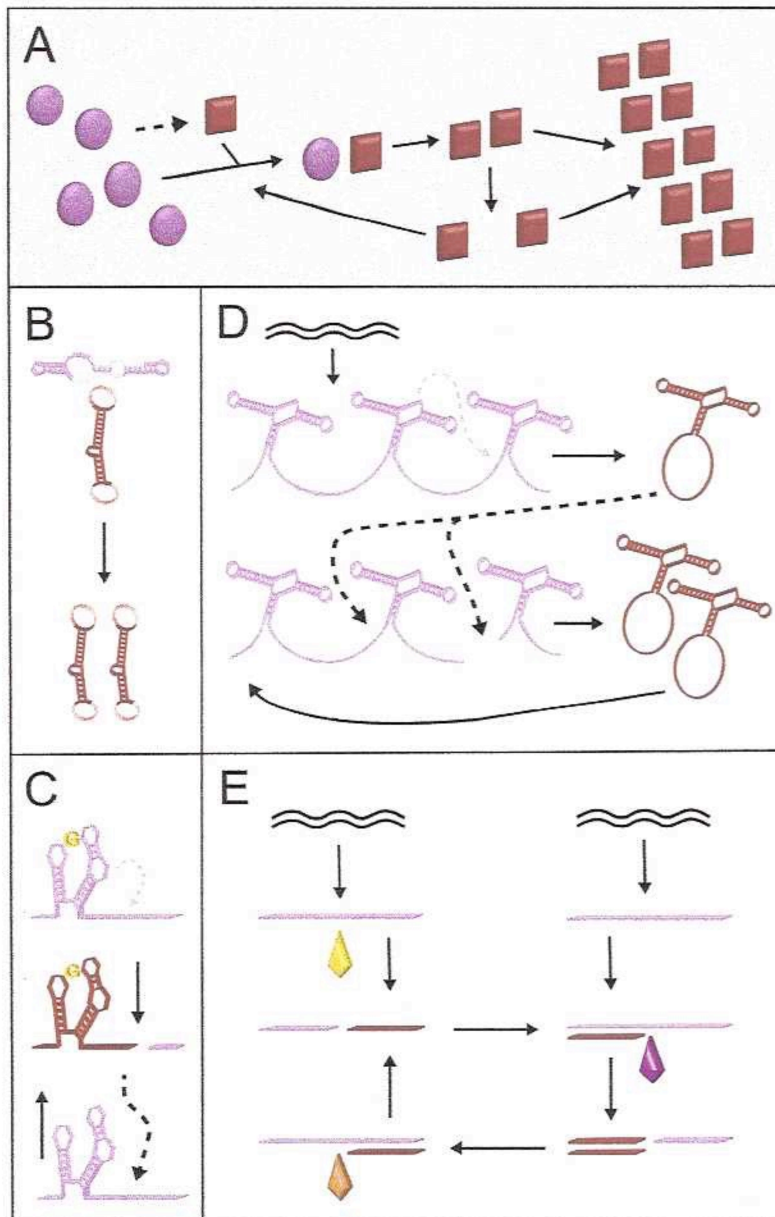

FIG 1 Protein prions and RNAs that may behave like prions. In all panels, the dark blue lines and shapes indicate the benign forms of proteins or RNAs, red lines and shapes indicate the prion forms, solid arrowed lines indicate the direction of the reaction, and broken arrowed lines indicate the autocatalytic cleavages (gray for the relatively inefficient reactions and black for the more efficient ones). (A) A general scheme of protein prion induction and propagation. The benign form of a protein is converted into the prion form only rarely and spontaneously (left), but once formed, it is able to turn more copies of the benign form into the prion form (center), and in many cases to form aggregates in the cell (right). (B) The "Viennese prion." (C) A putative prion-like derivative of the *glmS* ribozyme/riboswitch. The GlcN6P-dependent version described in the text is shown. The green letter G indicates the GlcN6P ligand. The engineered ribozyme requires the presence of a ligand for activity but cleaves with reduced efficiency when acting in *cis*, and with relatively high efficiency when acting in *trans*. (D) A putative viroid-derived system engineered to possess prion-like properties. The concatemeric plus-strand viroid RNA is transcribed from the integrated DNA copies (black wavy lines). The engineered HHR region within the viroid RNA processes the concatemer into the unit-length viroids with reduced efficiency when acting in *cis*, and with relatively high efficiency when acting in *trans*. (E) Prion-like properties of the ping-pong mechanism of piRNA production. The genomic copies of piRNA clusters and evolutionarily related active transposon copies are shown by black wavy lines, and the enzymes from different protein families that process the piRNA precursors into the mature piRNAs are depicted as gemstones of various colors.

conformation either spontaneously and rarely, or with higher frequency by the action of another copy of a prion; and the prion-associated phenotype is transmitted between cells together with the prion form of the protein. In addition, three operational criteria have been proposed that help to identify candidate prions (22): (i) prions in yeast may be revealed by the fact that the rate of their emergence is increased when the cognate gene is overexpressed, (ii) prions in yeast are often reversibly curable, and (iii) prion phenotype mimics the loss-of-function phenotype of the cognate gene (the last, however, is true only if the prion form is the one in which the normal function of a protein is inactivated; if prion is the active form of a protein, this will manifest as a gain of function).

The molecular properties of the benign and prion protein forms in fungi are of particular interest in the context of the new definition of a prion. Though many fungal prions contain protein segments that under some circumstances can form specific amyloid-like, proteinase K-resistant arrangements of parallel in-register β-sheets *in vivo* and *in vitro*, this has not been shown for all prions. Moreover, yeast prions do not have to form amyloid at all. Such is the case of the vacuolar protease B (PrB, encoded by the *prb1* gene), which is synthesized as an inactive zymogen precursor that must be activated through the sequential removal of regions at both termini by another protease (protease A/Pep4p) and by a mature copy of PrB. In the strains where Pep4p is deleted, mature Prb1p can in rare cases activate its own precursor. This conversion of an inactive to active form can propagate within a cell and pass between cells, satisfying the definition of a prion (23, 24); in this case, the prion, called [β]—no connection to β-sheets in amyloids—is the same as the active form of protease B. In the absence of Pep4p, [β] is required for survival in stationary phase and for meiotic sporulation. With the admittance of [β] to the class of prions, the definition of a prion must be modified again, to allow that the chemical composition of the prion and nonprion forms does not have to be exactly the same—protein processing may be permitted. This should not be seen as an unprecedented departure from the prion conventions, given that the posttranslational processing events, such as glycosylation, play a role in the expression of the infectious phenotypes in mammalian PrP (25). The case of prion [β] argues for omitting the amyloid formation from the list of essential properties of a prion. Two other recently described yeast prions, [SMAUG+], the product of the *Vts1* gene, and [ESI+], the product of the *Snt1* gene, also do not appear to form amyloids, though both may form other kinds of aggregates (26, 27).

Can there be RNA prions? We propose to take another step in expanding the definition of a prion and admit the possibility of the prion-like behavior in another class of biopolymers, namely, RNA. Even though RNA for a long time has been seen primarily as a carrier of genetic information or facilitator of protein synthesis, it is now clear that RNA molecules may perform catalytic and regulatory functions that do not involve encoding proteins but instead rely on the enzymatic activity, ligand-binding ability, or capacity for dynamic structural rearrangements of RNA itself. In the rest of this paper, we outline the definition of an RNA prion and review several classes of RNA that may come close to satisfying this definition.

Most criteria for a protein-based prion can be generalized for RNA (Table 1, "RNA prion definition in this work"): an RNA prion is encoded by the cellular gene but inherited extrachromosomally; it has a phenotype that is due to the function of the RNA itself, not of its encoded protein, if such protein exists; and the phenotype mediated by an RNA prion is inducible and transmissible. RNA prions have two forms, a benign and a phenotype-causing one; analogously to the case of the protein prion [β], conversion between the benign and prion form of RNA may involve RNA processing. The benign form may undergo a rare conversion to the prion form, perhaps stimulated by stress or other external factors. When the prion form is already present, the rate of conversion of benign copies to prion form increases.

Several of the above properties have been observed in some naturally occurring or computationally designed RNA molecules. We next review four cases of the RNAs that

may come close to satisfying the expanded prion definition. We note that, as with yeast protein prions, a neurological phenotype is not required for an RNA prion, and neither is amyloid formation.

"Viennese prion." Stefan Badelt and coworkers have presented the results of a computational design of an RNA satisfying the following condition: the molecule must be bistable, i.e., it must preferentially be in one structural state when it is a monomer and preferentially adopt another structural state when it is in a dimer (28). Thermodynamic calculations have been done to determine the stability of both forms and ensure that they are separated by a ridge on the folding landscape, without any local free energy minima close to either of the two stable conformations. The designed putative RNA prion (the authors modestly called it "an RNA with prion-like properties") is a circular RNA consisting of 49 nucleotides, which exhibits an extensive pairing in both conformations. One form, called S_1, persists as a monomer, while the other, S_2, has two stem-loops that are likely to hybridize via a kissing-loop interaction when present on two different molecules, stabilizing the S_2 form in a dimer. The two loops are sterically unlikely to interact when they are in the same molecule, but either of the loops can enter a kissing interaction with the complementary sequence on another copy of S_1. Such a complementary region is partly occluded by alternative base pairing within S_1, but its interaction with S_2 melts this alternative pairing, forcing S_1 to change its conformation into S_2 (Table 1 and Fig. 1). These properties, once realized in a physical RNA molecule synthesized *in vitro*, will satisfy several criteria of an RNA prion; of course, biological and genetic criteria, such as the phenotype caused by this "Viennese prion," as well as the conditions of its induction and curing, may only be considered after introduction of such a construct into a living cell.

Self-processing riboswitches: the *glmS* example. Riboswitches are structured regions that are found in the noncoding portions of mRNAs in all three domains of life. In bacterial and archaeal mRNAs, riboswitches are more often located in the 5′ untranslated regions (UTRs), and in eukaryotic mRNAs, they are found mostly in the 3′ untranslated regions and in introns. Many riboswitches regulate gene expression, typically by binding small metabolites and inducing changes in the synthesis or stability of the mRNAs in which they are embedded (29, 30). In an elaboration of this theme, a riboswitch from the 5′ UTR of the mRNA of the *glmS* gene, conserved in many Gram-positive bacteria, was shown to self-cleave in the presence of glucosamine-6-phosphate (GlcN6P). The *glmS* open reading frame encodes glutamine-fructose-6-phosphate amidotransferase, which is the terminal enzyme of GlcN6P synthesis. Self-cleavage of the 5′ UTR in the *glmS* mRNA exposes the transcript to degradation, therefore ensuring the shutdown of metabolite production when it has accumulated in the cell (31).

Various derivatives of the *glmS* riboswitch-ribozyme have been designed experimentally or selected in *in vivo* evolution experiments, including the forms active in the absence of GlcN6P and a variant efficiently cleaving its own copies in *trans* (32, 33). It is conceivable that these properties could be combined to generate new RNAs, which may display prion-like behavior. One version of such a putative RNA prion would be active in the presence of GlcN6P; in such a case, one could engineer an embedded copy of the ribozyme that cleaves inefficiently in *cis* but releases a product that cleaves the embedded copies of itself more efficiently in *trans*. The RNA prion then would be inducible by GlcN6P, the prion infection would propagate in the cell, and the RNA prions transferred into another cell would initiate prion formation there, as long as GlcN6P is present (Table 1 and Fig. 1). This would provide the same catabolite repression phenotype as the one mediated in *cis* by the unmodified *glmS* ribozyme-riboswitch, except for perhaps different response kinetics. In a GlcN6P-independent version of *glmS*, the released copy of the ribozyme could be engineered to be more active than the embedded one; this would be essentially a toxic prion, similar to the TSE prions and many yeast protein-based prions.

Viroids and viroid-like elements. By the end of the 1960s, Theodor O. Diener characterized the first of a new class of plant pathogens constituted by a naked small circular covalently closed RNA molecule (34). Since that seminal discovery, about 45 different viroid species have been described and classified into two taxonomic families according to sequence similarity and functional properties (35). The mature circular form of viroids (which superficially resembles the secondary structure of the S_1 form of the "Viennese prion" described above, though viroid genomic RNAs are longer) is infectious and transmissible between cells and organisms. Members of the larger *Pospiviroidae* family replicate in the nucleus of susceptible cells via a rolling-circle mechanism, relying on the cell for the three enzymatic activities required for the replication cycle: a DNA-dependent RNA polymerase as replicase, an endonuclease to process the oligomeric intermediates into monomeric genomes, and a ligase to close these monomers into mature circular molecules (36). The members of the smaller *Avsunviroidae* family replicate in the chloroplast and also require the replicase and ligase activities from the cell, but they do not require a cellular endonuclease, as they all encode, in genomic and antigenomic strands, self-cleaving hammerhead ribozymes (HHRs), which process the oligomers into genomic monomers.

The HHR also has been described in other viroid-like molecules, such as the viroid-like satellites of nepoviruses and luteoviruses (also known as virusoids) and human hepatitis δ virus RNA. Interestingly, several tandem cDNA copies of a viroid-like molecule known as *Carnation small viroid-like RNA* (CarSV) are embedded into the DNA genome of a plant pararetrovirus, *Carnation etched ring virus*, and, via the integrated copies of this virus, have made their way into the genomes of carnation plants (37–39), satisfying the criterion of a genome-encoded factor. Unlike most bona fide viroids, these viroid-like molecules require the assistance of a helper virus to ensure their reproduction and transmission among hosts.

The HHR is composed of a catalytic core of conserved nucleotides flanked by three helices, two of which are involved in essential tertiary interactions that facilitate the self-catalysis of the phosphodiester backbone (40). In recent years, an increasing number of noncoding circular RNAs, varying in size between 100 and 1,000 nucleotides, have been reported in both plants and animals; all these genetic elements contain self-cleaving functional HHRs and have been called "retrozymes" and suggested to have evolved from Penelope-like retroelements which also harbor HHRs (34, 48).

It has been established long ago that "prions are not viroids," in the specific sense that there is no small viroid-like RNA associated with the protein fraction that transmits TSE, and that various inactivation experiments with mammalian infectious prions suggest the pattern of sensitivity typical of a protein, not of viroid RNA (4). However, if the definition of prion is extended to genetic elements made of RNA, it becomes evident that some real-life viroids of the *Avsunviroidae* family and viroid-like molecules containing HHR either display or can be engineered to display several prion-like features.

During the replication cycle of HHR viroids, cleavage turns the nontransmissible RNA multimers of the genomic-strand RNA into the infectious entity, the unit-length circular RNA, thus satisfying the requirement of the self-mediated conversion from the benign to prion form. The wild-type viroid HHRs are inactive when in monomers, but synthetic derivatives of the hammerhead ribozymes that are able to cleave in *trans*, usually at a reduced rate, have been obtained (41–43, 48).

A system consisting of a genomically integrated concatemer of a viroid-like cDNA, engineered to be expressed in an inducible fashion but, as in the case of CarSV, unable to replicate autonomously, would go a long way toward satisfying an RNA prion definition (Table 1). Such a system, although derived from a viroid, would not require RNA replication for the infectivity and prion-like behavior—the transcription of the gene integrated in the host genome will suffice, just as in the case of the protein prions. To confer even more prion properties to a CarSV-like RNA, its relative rates of cleavage in *cis* and in *trans* would have to be reversed, so that a noninfectious multimeric transcript is processed into the unit-length forms in *cis* only rarely, but the mature viroid

would cleave in *trans* more efficiently (Fig. 1). In such a design, the prion property of the entire system would be maintained by an HHR-containing RNA element that propagates itself by cutting the benign multimeric RNA forms that the cell produces by transcribing the integrated concatemers; the products of the processing are the unit-length viroid-like RNAs. It is likely that the knowledge about viroid-encoded HHRs is already sufficient to engineer such a construct, which could serve as another model of an RNA prion *in vivo*.

Small piRNAs produced by ping-pong mechanism. The PIWI-interacting RNA (piRNA) pathway is a gene silencing system that is thought to protect animal germ line cells from the deleterious effects of the activity of transposable elements (TEs). Experiments in the fruit fly have shown that the gamete development is dependent on the expression of a specific fraction of small (23- to 30-nucleotide) RNAs, which are complementary to transposable elements and some other genome repeats. Such small RNAs are also found in the reproductive tissues of vertebrate animals, and in all species, they are associated with the members of a particular clade of the Argonaute proteins, the PIWI family, from which the name piRNA is derived (44). Multiple piRNAs are genomically encoded, typically by piRNA clusters, which are the loci of deleted or nested copies of DNA transposons that have lost the ability to transpose. Transcription of either one or both DNA strands in these clusters produces piRNA precursors, which are bound by a cascade of RNA-binding and RNA-compartmentalizing factors and then processed into the mature "primary" piRNAs by at least two RNases. The primary piRNAs associate with three proteins from the PIWI clade, and interestingly, the sequences bound to the fruit fly PIWI-clade proteins Piwi and Aubergine on the one hand and Ago3 protein on the other hand show orientation bias, different terminal sequences, and 10-nucleotide overlap (44, 45). These and other observations led to the idea of the ping-pong model, in which the piRNAs derived from the transcripts from one DNA strand aid the formation of complementary overlapping piRNAs, which are produced from the transcripts encoded by another strand. Since piRNA clusters are derived from the defective TEs, the full-length transcripts from the nondefective TEs are also targeted, and ping-pong amplification simultaneously generates more piRNA and silences the target TEs by inactivating their transcripts (as well as by other mechanisms, reviewed in reference 45, that are not considered here).

If we stretch the RNA prion framework somewhat further than in the three previous examples, the system of piRNA production may reveal certain features in common with prions. In this view, the long precursor transcripts of the primary piRNA loci may be seen as the inactive form of the prion; with RNA processing being permitted as part of the prion lifestyle, the piRNA would be the active form of a prion beneficial to the cell (Table 1 and Fig. 1). Primary piRNAs induce the production of more copies of themselves; the amplified secondary piRNAs can be transmitted between cells and, at least in the nematode *Caenorhabditis elegans*, between generations (46).

Here, we examined the plausibility of expanding the definition of a prion beyond what has been done before, to include the inducible and transmissible RNA agents that can autocatalytically convert themselves from inactive to active form. We have shown that several designed or naturally occurring classes of RNAs come close to satisfying such a definition. One might disagree with the appropriation of the term "prion" to something that is not made of protein (note, however, that "prion" as an acronym of "proteinaceous infectious agent" is itself malformed—see discussion in reference 47). Linguistic concerns notwithstanding, we think that adding a new dimension to the concept of prion helps to assess the robustness of the concept itself, as well as its applicability to various phenomena in molecular biology. In addition, the analysis presented above immediately suggests several new modalities with interesting properties, which may be constructed and tested by synthetic biologists.

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