Biosafety considerations of open air genetic engineering. An analysis of the New Zealand Environmental Protection Authority's reasons for not classifying organisms treated with double-stranded RNA as genetically modified or new organisms

Jack A. Heinemann
School of Biological Sciences, Centre for Integrative Research in Biosafety, Centre for Integrative Ecology, University of Canterbury, Christchurch, New Zealand
jack.heinemann@canterbury.ac.nz

Corresponding
Jack A. Heinemann
School of Biological Sciences, Centre for Integrative Research in Biosafety, Centre for Integrative Ecology, University of Canterbury, Christchurch, New Zealand
jack.heinemann@canterbury.ac.nz
+64 33695597
ORCID 0000-0002-9074-9052

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Abstract

The New Zealand Environmental Protection Authority (EPA) issued a Decision that makes the use of externally applied double-stranded (ds)RNA molecules on eukaryotic cells or organisms technically out of scope of legislation on new organisms, because in its view the treatment does not create new or genetically modified organisms. The Decision rests on the EPA's conclusion that dsRNA is not heritable and therefore treatments using dsRNA do not modify genes or other genetic material. I found from an independent review of the literature on the topic that each of the major scientific justifications relied upon by the EPA to conclude that exposures to exogenous sources of dsRNA were out of legislative scope was based on either an inaccurate interpretation or failure to consult the research literature on all types of eukaryotes. The Decision also has not taken into account the unique eukaryotic biodiversity of the country. The safe use of RNA-based technology holds promise for addressing complex and persistent challenges in public health, agriculture and conservation. However, the EPA removed regulatory oversight that could prevent the accidental release of viral genes or genomes by failing to restrict the source or means of modifying the dsRNA.

keywords: RNA interference, biosafety regulation, dsRNA, gene silencing, genetically modified organisms, eGE

Introduction

In May 2018 the Decision-Making Committee of the New Zealand Environmental Protection Authority (EPA) published a 4-page announcement concluding that eukaryotic cells or organisms treated with double-stranded (ds)RNA are not new organisms (EPA 2018a). This critical determination has implications for the regulation of new biotechnologies in New Zealand because there is growing interest in the development of dsRNA for use in medicine (Lam 2012) and agriculture, such as for pest control (Sammons et al. 2011; Van et al. 2011; Whyard et al. 2011; Huang et al. 2018).

Environmental biotechnologies are regulated by the EPA under the 1996 Hazardous Substances and New Organisms (HSNO) Act (1996). The EPA can regulate on the basis that an organism that has been treated with dsRNA is a new organism, or instead categorize RNA as a chemical that could be a hazardous substance.

RNA is not now and unlikely ever to be listed as a hazardous substance. This is deduced from the observation that none of the terms RNA, dsRNA, ribonucleic acid,

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or siRNA return anything in a search of the EPA's databases: "Approved hazardous substances with controls", "Chemical Classification and Information Database", or "New Zealand Inventory of Chemicals." Moreover, the Ministry of Primary Industries places RNA in the "Neglible Risk Register" (MPI 2018).

In which case if dsRNA-treated organisms are to be regulated at all, they must be under the country's biosecurity laws* directed at pathogens and pests, or as new organisms by the HSNO Act. A new organism may be in a species or sub-species new to New Zealand and/or be a genetically modified organism of any species (full definition in Supplemental Material). Here I analyze the routine case where an organism that is not new (or considered to be a biosecurity threat) is treated with dsRNA, and whether that treatment results in the organism being a new organism under the HSNO Act, by creating a genetically modified organism.

The HSNO Act says that a "genetically modified organism means, unless expressly provided otherwise by regulations, any organism in which any of the genes or other genetic material—(a) have been modified by *in vitro* techniques; or (b) are inherited or otherwise derived, through any number of replications, from any genes or other genetic material which has been modified by *in vitro* techniques" (1996).

New Zealand is harmonized to the Cartagena Protocol on Biosafety (the Protocol) (CBD) through its HSNO Act. The Protocol is an international treaty on the transboundary movement of products of modern biotechnology, including living genetically modified organisms. The Protocol does not apply to food and pharmaceutical products that are unable to survive in the environment. The Protocol definition of a living modified organism is "any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology." The Protocol definition of modern biotechnology is "the application of: a. *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles, or b. Fusion of cells beyond the taxonomic family, that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection."

Binding international agreements such as the Cartagena Protocol create a network of countries with shared obligations. Domestic legislation or *sui generis* authority is required by each country to meet its obligations. As a result, legal frameworks arise

* Biosecurity is overseen by the Ministry of Primary Industries in New Zealand, and refers to "stopping pests and diseases at the border, before they get to New Zealand, and eradicating or managing the impact of those already here." MPI Biosecurity. Retrieved 26 June 2018 https://www.mpi.govt.nz/law-and-policy/legal-overviews/biosecurity/

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that tend to have similarities, and challenges to them in one member country are frequently encountered also by others (Kershen 2015). An example is the use of techniques referred to as gene editing. New Zealand was the first country in the world to establish its legal view on gene editing techniques (Kershen 2015). These questions were and are discussed in many countries, but notably all countries that are bound to the Protocol and its language will be affected by the decisions member countries make. In 2018 the European Court of Justice came to determinations similar to New Zealand's.

Not all countries are members of the Cartagena Protocol on Biosafety. However, the Protocol achieved the threshold of membership that brought it into force and non-member countries must respect the right of member countries to legislate on the matter of transboundary movement of products of modern biotechnology. Non-member countries that arrive at different definitions still must respect the biosafety laws of member countries when trading in such products provided that the laws are harmonized to the Protocol. New Zealand's instrument is its HSNO Act.

HSNO Act has language similar to—but consequentially different from—the Protocol. Both make reference to *in vitro* techniques, but the Protocol emphasizes the use of nucleic acids (eg dsRNA is a nucleic acid) whereas the HSNO Act emphasizes modification of genes and other genetic material. dsRNA can be relevant to New Zealand law if its use modifies genes or other genetic material, for example by being a type of mutagen or by becoming part of the genome as in the use of transgenes. The HSNO Act defines neither genes nor genetic material, so whether or not dsRNA treatments are in its scope is not made clear from definitions.

The Convention on Biological Diversity provides some guidance by defining genetic material (CBD). This is the parent treaty to the Protocol. New Zealand is a Party to both. The Convention on Biological Diversity defines genetic material as "any material of plant, animal, microbial or other origin containing functional units of heredity" and genetic resources as "genetic material of actual or potential value." This is also how the International Treaty on Plant Genetic Resources for Food and Agriculture defines both plant genetic resources and plant genetic material (ITPGRFA).

Therefore, genetic resources are a special kind of genetic material, one that has actual or potential value. Genetic resources are described as such things as organisms, seeds, zygotes and cuttings (Europa ; FAO). They include the nucleic acids such as DNA but are not exclusive to them. None of these international or domestic legal instruments defines the terms "genes" or "functional units of heredity." Nor do these instruments or domestic law define the term "modify."

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EPA received an application to determine if "eukaryotic cells treated with synthetic double stranded RNA" were new organisms (Trought 2018). As the regulatory authority, the EPA makes two kinds of decisions (Fig. 1). The first kind is whether or not a regulated organism or substance is safe to use or how it could be safe to use. This follows from a mandatory risk assessment. The second kind, which is the kind relevant to this article, is whether an organism is regulated. If it is not regulated, then a risk assessment by the Authority will not be done regardless of whether or not a risk assessment would be useful. Therefore, the focus of this article is not on what potential hazards may arise from the use of dsRNA, or how to assess and mitigate putative hazards, which are covered elsewhere (eg Refs Heinemann et al. 2013; FIFRA 2014). The focus of this article is on the scientific information used by the EPA to determine that dsRNA treatments do not result in the kinds of effects that make an organism new or genetically modified.

I will explore the scope of the Decision and then analyze the major arguments and information sources used by the Decision-Making Committee and EPA staff. The main reason for determining that treatments using dsRNA did not result in new organisms was that externally applied (exo-)dsRNA is not inherited by the organism (Fig. 1). The Committee identified several factors that prevented inheritance. These factors were that exo-dsRNA molecules could not enter the nucleus, they are not reverse transcribed into DNA, and for both of these reasons they therefore could not integrate into the DNA of the genome and modify it, and by implication only DNA and only the DNA in the nucleus was heritable genetic material (paragraph 4.6 of Ref EPA 2018a).

The decision

The Committee's Decision in context is about the use of exo-dsRNA for the purpose of causing RNAi (Box 1). As the nature and source of the dsRNA applied as exo-siRNA is undefined by the EPA in its Decision, I will often use the term exo-dsRNA as a more generic description than exo-siRNA in this analysis.

Analysis of the Decision is made more complicated because the EPA Decision-Making Committee described the application in various, and significantly different, ways (Table S1) and different to the descriptions provided by either the applicant (Trought 2018) or EPA staff (EPA 2018b).

Moreover, the Decision does not preclude the use of dsRNA that might result in other kinds of effects either inadvertently or on purpose. dsRNA (and RNA in general) can have effects on organisms (eg Refs Kalluri & Kanasaki 2008; Kleinman

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et al. 2008) other than RNAi, including heritable effects that are not associated with RNAi. After all, RNA is itself the material of genes, such as in RNA viruses and retroviruses. These viruses can have either dsRNA or single-stranded RNA genomes. They replicate independently of human intervention once inside a eukaryotic cell. Genetic information can pass from them to a DNA genome, and back.

Research on dsRNA-mediated gene regulation has advanced rapidly, but there is much still unknown about its biochemistry, even in the relatively few model organisms in which it has been studied (Djupedal & Ekwall 2009; Ghildiyal & Zamore 2009). Already it is clear that dsRNA-mediated gene regulation biochemistry is different between plants, animals, and fungi (Ghildiyal & Zamore 2009). Perhaps even more importantly, almost nothing is known about RNAi pathways in species unique to New Zealand. According to the Encyclopedia of New Zealand, Te Ara, "over 80% of the 2,500 species of native conifers, flowering plants and ferns are found nowhere else." "The best guess of the numbers of land-based native plants and animals is around 70,000 species. Insects and fungi dominate, each having an estimated 20,000 species – many are not yet described" (Manatū Taonga Ministry for Culture and Heritage).

Other exposures

The Decision could remove any need to notify the public of their potential exposures. The various kinds of exposures are through spray drift of dsRNA-based pesticides or brushing against treated plants, and ingestion of treated food items. Different exposure pathways – ingestion, inhalation or contact – have been studied at different levels. While the most research involves ingestion exposure and so far suggests that unmodified dsRNAs are unlikely to cause an effect in humans, this is still not fully certain (FIFRA 2014). The other exposure pathways have received very little attention (Heinemann et al. 2013; FIFRA 2014).

Hypothetical uses on post-harvest or retail foods include for the purposes of delaying ripening or spoilage. For example, genetically modified tomatoes were engineered to produce dsRNA to silence the expression of 1-aminopropane-1-carboxylate synthase, the rate-limiting enzyme in the production of the ripening hormone ethylene. The expression of dsRNA was controlled by a promoter that was mainly active late in development so as to not interfere with the production of ethylene at other stages of fruit development (Gupta et al. 2013). The effects of silencing at the wrong time could alternatively be avoided by spraying exo-dsRNA on harvested but unripe tomatoes. Other approaches are to use topically applied exo-dsRNA to silence genes that are receptors of ethylene (Deikman et al. 2017).

In these cases the primary concern would be the quality and purity of the dsRNA active ingredient. As discussed in more detail later in this analysis, the EPA Decision

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was agnostic to methods of preparing the dsRNA, or the size of the RNA molecules that might be used. Contaminants of dsRNA preparations from whole cells or tissues could include mRNA that might upon entry to cells be used to produce proteins that could be a source of allergens or toxins, and some RNA molecules could be substantial if not complete viral genomes.

Potential unavoidable exposures of non-eukaryotic organisms, such as bacteria, to RNA that could result in effects other than RNAi also were not evaluated in the documents released by EPA. Open air applications of dsRNA would result in exposures to non-target organisms, such as bacteria, including on the surface of target organisms.

Small RNA molecules are gene regulatory agents in bacteria, but do not use the biochemistry of RNAi (Papenfort & Vanderpool 2015; Mars et al. 2016). The intercellular trafficking of regulatory RNA molecules indicates that exo-RNA is relevant to their biology too (Sjöström et al. 2015).

Regulatory RNA in bacteria influences the transition from planktonic to biofilm growth (Ashley et al. 2017) and colonization of the intestine by pathogens (Han et al. 2017). RNA molecules serve as guides for the action of the nuclease Cas9 in the CRISPR/Cas9 system (Marraffini & Sontheimer 2010). Exo-dsRNA secreted by intestinal cells has been implicated in adjusting the growth rate of different species of bacteria in the human gut (Liu et al. 2016). Nowhere in nature, and even rarely in the laboratory, would eukaryotic organisms (as opposed to tissue culture cells) be free of prokaryotes.

Kinds of RNA molecules and treatments

The Committee did not address the physical description of the dsRNA in the approved treatments. The applicant sought permission to use “synthetic” dsRNA, restricted as well to those that would cause a temporary effect on the “activity of the complementary RNA” (Trought 2018). Although siRNAs tend to get processed down to <30 nucleotides, the Decision is not restricted to externally applied dsRNA molecules of <30 nucleotides. The dsRNA molecules possibly could be further chemically modified to mimic other classes of RNAs such as piRNAs (Ghildiyal & Zamore 2009) or to affect their longevity and stability (Table 1). At least 128 different modifications have been reported so far in the literature (Dar et al. 2016; siRNAmod 2018) and many synthesized siRNAs can be routinely ordered with modifications (Bioland 2018; Sigma 2018).

Moreover, dsRNA or single-stranded RNA may be expressed in bacteria and packaged *in vivo* into virus-like particles (Arhancet et al. 2016; Killmer et al. 2016). These techniques can increase stability of the RNA, allow selective release of the

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RNA cargo, and also allow for significant increases in quantities of RNA that may be produced and purified.

Beyond modifications to the dsRNA molecules are the formulations or materials, such as cell penetrating proteins (Numata et al. 2014), that might be used to improve penetrance. The Decision imposed no restriction on method or material for causing the dsRNA to be taken up by organisms.

The Decision makes it possible to use dsRNA made or amplified from natural sources, such as cellular material, which could contain contaminating active RNA or retro viruses (Ngo et al. 2017). Without the requirement for the EPA to review any externally applied dsRNA, treating a eukaryotic cell with either dsRNA corresponding to all or most of a messenger RNA or most of an RNA virus genome would be allowed. Responsible use of dsRNA for treating eukaryotes would unlikely include the purposeful amplification or modification of RNA viruses. However, the Decision specifically removes EPA from responsibility for protecting against inadvertent amplification of RNA viruses by saying "it was not necessary to consider whether *in vitro* techniques were involved." This is surprising given the accessibility of both genetic databases and recent revelations that a poxvirus was assembled by purchasing the component DNA fragments through "the mail" and the expectation that portable synthesizing equipment will be more common in the future (Sharples 2017). Even well intentioned molecular biologists, not to mention citizen scientists, could use molecules of unknown potential to replicate in some eukaryotes.

Commercial applications demonstrate heritability

Interestingly the EPA decision that exo-dsRNA treatments are not heritable through modification of genes or other genetic material directly contradicts industry intellectual property rights claims (Fillatti et al. 2012; Crawford et al. 2014; Deikman et al. 2017). In the patent "Methods and compositions for introducing nucleic acids into plants" including dsRNA, the claim is for both treated organisms and their progeny:

"Several embodiments include *progeny seed or propagatable plant part* of such plants, and commodity products produced from such plants…wherein the modification of the target gene is non-heritable silencing of the target gene, *or heritable or epigenetic silencing of the target gene*, or a change in the nucleotide sequence of the target gene; embodiments include *the directly regenerated plant* exhibiting modification of the target gene and *plants of subsequent generations grown from the directly regenerated plant* and exhibiting modification of the target gene" (emphasis added to Ref Huang et al. 2018).

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The type of patent used is a utility rather than plant variety patent and extends to the ownership of organisms and future generations of organisms treated with exogenous dsRNA similarly to how utility patents claim the use of genetically modified organisms.

"Several embodiments include a plant or a field of plants treated by a method, composition, or apparatus described herein, wherein the plant exhibits a desirable phenotype (such as improved yield, improved tolerance of biotic or abiotic stress, improved resistance to disease, improved herbicide susceptibility, improved herbicide resistance, and modified nutrient content) resulting from the treatment and when compared to an untreated plant. Several embodiments include progeny seed or propagatable plant part of such plants, and commodity products produced from such plants" (Huang et al. 2018).

The maker of the dsRNA would apparently own an organism because it was exposed to the dsRNA, potentially including entire fields of conventional crops or long-lived trees and their seeds that have never been modified by insertion of DNA.

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272 **Box 1: The Science of RNAi**

RNA interference (RNAi) is a form of gene regulation in eukaryotes with many potential biotechnological applications being discussed by regulators worldwide. (Heinemann et al. 2013; FIFRA 2014) RNAi pathways are found in nearly all eukaryotes (Agrawal et al. 2003). RNAi is often referred to as gene silencing, but it also is known to sometimes cause an increase in the expression of genes (Carthew & Sontheimer 2009; Kim et al. 2009).

RNAi results in what is called post-transcriptional gene silencing and transcriptional gene silencing (Kalinava et al. 2018). Post-transcriptional gene silencing occurs through dsRNA-mediated endonucleolytic cleavage or exonucleolytic destruction of the transcript or inhibition of translation of the transcript (Carthew & Sontheimer 2009; Rechavi 2014). In some organisms, dsRNA-mediated transcriptional gene silencing is caused by the modification of histones and DNA, while in others it may only be modification of histones, resulting in formation of heterochromatin and a decrease in transcription (Matzke & Birchler 2005).

The nomenclature for dsRNAs is expansive, but the main classes include siRNA (short-inhibitory RNA), miRNA (microRNA) and piwi-interacting RNAs (piRNA) (Carthew & Sontheimer 2009; Ghildiyal & Zamore 2009). These types are foundation substrates in biochemical pathways involving Argonaute proteins that cause RNAi.

The nomenclature should be used as an indicative guide to biogenesis of the dsRNA, but not the activity of the active form. This is because regardless of their source, dsRNAs share the same pathways in the cell (Ghildiyal & Zamore 2009). “For example, siRNA is able to mimic microRNA (miRNA) to inhibit translation or elicit the degradation of [messenger RNAs] with partial sequence complementarity” (Zhou et al. 2014).

All three active forms derive from longer dsRNAs. Cytoplasmic Dicer converts the longer form of siRNA and miRNA into the active form of about 21-23 nucleotides. Argonaute proteins bind to the RNA and carry out the regulatory functions (Carthew & Sontheimer 2009). Drosha (or Dcl1) acts in the nucleus to process pri-miRNA into pre-miRNA, which after transport to the cytoplasm is further processed to miRNA by Dicer (Kim et al. 2009).

Once associated with the Argonaute proteins, one strand of the dsRNA molecule is degraded and the other serves to guide the protein complex to its target. Some eukaryotic species have Argonaute proteins that can bind either miRNA or siRNA, and some that specialize in one or the other, while other species have Argonaute proteins that distinguish between miRNA and siRNA based on the structural features of the dsRNA.

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273 **Box 1: continued**

It is the Argonaute proteins that determine the mechanism of silencing (Rechavi 2014; Rankin 2015). Some Argonaute proteins, such as AGO2 in humans, have an endonuclease activity called slicer. These complexes cleave the target messenger RNA molecule. Human Argonaute proteins AGO1-4 and AGO1 of *Drosophila melanogaster* are examples that cause translational inhibition or degradation of the target transcript through exonucleolytic decay. Ago1 of *Schizosaccharomyces pombe* and AGO4 and AGO6 of *Arabidopsis thaliana* are examples that cause transcriptional gene silencing through heterochromatin formation (Kim et al. 2009).

The dsRNA is sorted amongst competing Argonaute proteins according to the number of mismatches and bulges, not because particular dsRNAs are genetically determined to exclusively follow pathways dedicated to miRNA or siRNA (Ghildiyal & Zamore 2009). In *Drosophila*, AGO1 tends to favour duplexes with more bulges and mismatches and results in translation inhibition while AGO2 prefers duplexes with near perfect complementarity and results more often in messenger RNA cleavage (Ghildiyal & Zamore 2009). However, even these rules are different between animals such as *Drosophila* and plants (Ghildiyal & Zamore 2009) making it difficult to generalize for all eukaryotes. In short, intending a particular dsRNA to be an siRNA does not mean that it will be.

The binding strength of the guide strand and target influences the outcome of the interaction. The combination of near perfect antisense pairing between guide strand and target involving an Argonaute with slicer activity results in strand cleavage by an endonuclease activity (Massirer & Pasquinelli 2013). The larger the number of mismatches between the guide and target RNA, the more likely the silencing will be caused by exonucleolytic decay or translational inhibition (Massirer & Pasquinelli 2013).

While endo-siRNA, miRNA and piRNA may be born differently, they are not reliably distinguished by the silencing biochemistry. Both miRNA and piRNA arise from transcription of genomic DNA. Although this can also be true for siRNA, such as from transgenes or transposons (endo-siRNAs), the term is also often reserved for exo-siRNAs even if they have a hairpin structure. In general, miRNAs are not transcribed from the protein coding region of a gene and may have more mismatches with their targets. The converse is true for siRNAs. Thus, miRNA, piRNA and endo-siRNA all first appear in the nucleus and exo-siRNA does not (Carthew & Sontheimer 2009).

It is not possible to confidently extrapolate the outcome of exposure to exo-siRNA based on similarity of nomenclature to endo-siRNA. Because of differences between organisms and differentiated cell types, generalizations based even on the structure of the dsRNA molecule often fail.

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Exo-dsRNA is not confined to the cytoplasm

The Committee understood that exo-siRNA remains "solely as RNA molecules in the cell cytoplasm outside the nucleus" (EPA 2018a), consistent with advice received from staff (paragraph 2.9 of Ref EPA 2018b). Physical isolation of the genes and other genetic material in the nucleus would be a biological barrier to inheritance of exo-dsRNA that was confined to the cytoplasm (Fig. 1).

However, no such barrier can be relied upon to keep dsRNA out of the nucleus. As discussed in detail below, exo-dsRNA converted into siRNA is transported to the nucleus and causes transcriptional gene silencing in at least some eukaryotes.

Transport

Processed exo-dsRNAs may be conducted to the nucleus in association with a variety of proteins including Dicer and NRDE-3 (Various ; Mao et al. 2015). Already a decade ago researchers reported that "NRDE-3 binds siRNAs generated by RNA-dependent RNA polymerases [RdRP] acting on messenger RNA templates in the cytoplasm and redistributes to the nucleus" (Guang et al. 2008).

Djupedal and Ekwall (2009) writing about heterochromatin formation—which is specific to the chromosomes in the nucleus—said that: "Exogenous siRNAs are thus capable of stable and specific epigenetic regulation of target genes." Djupedal and Ekwall were cited in the underlying research provided by staff to the Committee (eg paragraph 2.9 of Ref EPA 2018b).

Carthew and Sontheimer (2009), also cited by EPA staff (eg paragraph 2.2 of Ref EPA 2018b), said that miRNA and exogenous siRNA are biochemically interchangeable once in the cytoplasm (Box 1). Their biochemistries overlap, and no clear distinction can be made in the kinds of silencing that they cause, further undermining certainty that externally applied dsRNA could be relied upon to stay out of the nucleus.

Carthew and Sontheimer (2009) do make a distinction between miRNA and siRNA. They mention that siRNAs but not miRNAs silence their own transcripts and when miRNA is made in the cell, it is modified to prevent re-entry into the nucleus. However, as noted by the authors, this distinction fails sometimes, and it does not apply to external dsRNA (Carthew & Sontheimer 2009).

Nuclear envelope

Cytoplasmic and nuclear contents are separated by the nuclear envelope and the perinuclear space. However, each cell cycle the nuclear envelope breaks down in eukaryotes with open mitosis, resulting in mixing with the cytoplasm (Gorlich &

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Kutay 1999; Smoyer & Jaspersen 2014). This cyclic breakdown provides the Argonaute protein-associated RNA access to the chromosomes (Li 2008). In animals at least, the nuclear envelope can also rupture, resulting in mixing of content (Hatch & Hetzer 2014). This pathway is exploited by parvoviruses as part of the infection cycle.

Reverse transcription

Among potential barriers to inheritance is that exo-siRNAs will not be reverse transcribed (Fig. 1). Unfortunately, neither the Decision nor Staff Advice provided references or analysis for the definitive existence of such a barrier.

Reverse transcriptase has the ability to synthesize a DNA molecule using an RNA molecule as a co-factor (template), similar to how DNA itself replicates using a DNA strand as a co-factor in DNA replication. Once a DNA strand has been synthesized by reverse transcriptase, that strand can serve as a co-factor in the synthesis of a complementary strand to produce a double-stranded DNA molecule.

A variety of enzymes commonly found in eukaryotes have reverse transcriptase activity (Goic et al. 2013). By some estimates, as much as 30% of the mammalian genome, and 10% of the human, was created by the action of reverse transcriptase activity originating from retroviruses (de Parseval et al. 2003). Reverse transcriptases are also routinely used in transcriptomics experiments, in the first step of amplification of the transcriptome, including amplification of small RNAs even as small as siRNAs (Dard-Dascot et al. 2018).

Reverse transcriptase requires a primer to initiate synthesis. A primer is another nucleic acid polymer, usually RNA (such as a dsRNA molecule called a tRNA), that provides a 3´OH group for strand extension. The primer may come from the secondary structure (eg a hairpin structure), as is common in precursors of siRNA. Alternatively, the primer is a second molecule that binds to the template strand. The primer gives the reverse transcriptase reaction specificity because it binds by complementarity to a target sequence. At least in the laboratory, it is possible for a reverse transcriptase reaction to proceed without the addition of any particular primer molecule because there are sufficient numbers of small RNA molecules naturally present in the cytoplasm to serve this purpose (Frech & Peterhans 1994).

It is uncertain whether all exo-dsRNA molecules could be substrates for reverse transcriptase, but it is unlikely that none could be. RNA from viruses can be captured by reverse transcriptase for conversion into DNA molecules and integration into chromosomal DNA, as well as by Dicer for production of siRNA

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(Goic et al. 2013). It has long been known that RNA elements can be converted into DNA by the action of reverse transcriptase in eukaryotes. For example, a DNA virus, that infects animals, evolved via recombination between a DNA virus, that infects plants, and an RNA virus, that infects animals (Gibbs & Weiller 1999). The process involved reverse transcriptase from a third virus acting on the animal RNA virus to convert an RNA genome into DNA.

Significantly, an enzyme from bacteria has been discovered that is able to reverse transcribe from RNA templates and create short DNA fragments that were subsequently recovered in the chromosome (Silas et al. 2016). The possibility that DNA molecules are generated *in vivo* using exo-dsRNA constructs is made even more plausible by this discovery because the bacterial enzyme is most closely related to the reverse transcriptase of retrotransposons found in eukaryotes.

Thus, under the right conditions reverse transcriptase is able to use exo-siRNA as a substrate. The Decision places no size or structural constraints on the exo-dsRNA that can be used and therefore does not preclude conversion to DNA.

Other DNA modifications caused by dsRNA

dsRNA can cause at least three other kinds of changes to DNA in the chromosomes of the nucleus of a cell independent of being reverse transcribed: DNA deletions (Matzke & Birchler 2005); changes in chromosome copy numbers (Khurana et al. 2018); and modification of nucleotides (Matzke & Birchler 2005).

Deletion

The eukaryote *Tetrahymenia thermophila* has an "RNAi-mediated process that directly alters DNA sequence organization" (Mochizuki & Gorovsky 2004). Approximately 12,000 DNA sequences, comprising 46 mega-bases, are deleted (Noto & Mochizuki 2017). DNA fragments removed from *Paramecium tetraurelia* chromosomes by a dsRNA-guided mechanism are ligated together to form an extra-chromosomal element that is transcribed and processed into more dsRNAs (Rechavi & Lev 2017). While this process has been described for endogenous dsRNAs, the example further demonstrates the difficulty in making generalizations about dsRNA effects on DNA.

dsRNA also causes heritable changes in DNA rearrangements in the eukaryote *Oxytricha trifallax.* These organisms have two nuclei in each cell. The somatic macronucleus contains the genes being actively transcribed in somatic cells. During development of the macronucleus, 95% of the germline genome is destroyed

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resulting in extensive fragmentation followed by permutations and inversions (Nowacki et al. 2008). RNA guides the rearrangement process. Exo-dsRNA that targeted these guides prevented reassembly of DNA fragments in the macronucleus (Nowacki et al. 2008).

Copy number

The number of copies of chromosomes in the macronucleus in the cells of *O. trifallax* is regulated by dsRNA. The number of duplicates of chromosomes in the macronucleus was shown to increase from exposure to exo-dsRNA (Khurana et al. 2018). The exposure did not noticeably alter gene expression, but the effects on chromosome number were dependent on Dicer and RdRP activity. Using antibodies that recognize DNA:RNA hybrid molecules, siRNAs were shown to directly associate with chromatin. Moreover, the exo-dsRNA effect on the copy number of the DNA chromosomes was heritable (Nowacki et al. 2010).

Modification

Modification of genetic material is caused by more than just changes to the primary sequence of DNA molecules through integration, deletion or mutagenesis. Transcriptional gene silencing is caused by chemical modifications in the form of methyl groups added to nucleotides and histones by RNA-directed DNA methylation, promoting heterochromatin formation (Djupedal & Ekwall 2009; Woodhouse et al. 2018). Methylation of DNA also influences RNA splicing patterns in insects, altering protein structure and diversity (Brevik et al. 2018).

Finally, methylation can also change mutation frequency because methylated cytosines deaminate to thymine, causing transition mutations. T:G mismatches are 10 times less likely to be repaired than other mismatches (Holliday & Grigg 1993). In both people and plants methylation tends to occur more in genes with naturally lower numbers of C residues, presumably because of historical deleterious transition mutations at these loci (Zilberman 2017). The outcome of the use of exo-dsRNA could be targeted mutagenesis in the eukaryotes that have RNA-directed DNA methylation pathways.

The modification of histones and nucleotides in genes passes through mitosis and meiosis (CGRFA 2015). Once methylation has occurred, it can be propagated independently of further stimulation by exogenous dsRNA. As Djupedal and Ekwall (2009), who also were cited by EPA staff, say: “It is easy to visualize how DNA methylation is inherited from mother cell to daughter cell considering that DNA replication is semi-conservative and the newly synthesized strand may be methylated with the ‘old’ strand as template. Likewise, half of the histones are partitioned to each DNA helix during S-phase, and may thereby guide histone

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modifications to newly incorporated histones. This would provide means for maintenance of the chromatin setting over cell divisions." This mechanism has been shown for both sexual and asexual reproduction of eukaryotes.

The examples above would fall well within the parameters of evidence that dsRNA causes modification of genes or other genetic material that is "capable of being inherited by the progeny of the organism, or…capable of causing a characteristic or trait that can be inherited" (EPA 2018b).

Genes are not confined to the nucleus

Even if it were the case that exo-dsRNA was confined to the cytoplasm, eukaryotes have genes there too. Cytoplasmic organelles called mitochondria and chloroplasts have DNA genomes. Separate from them, some eukaryotes have self-replicating DNA and RNA elements in the cytoplasm.

The eukaryotes *Kluyveromyces lactis, Pichia acacia* and *Debaryomyces robertsiae* host cytoplasmic linear DNA plasmids (Wickner 1986; Wickner & Edskes 2015). Large versions of these "virus-like elements" have all the genes necessary for replication and maintaince, and may provide some of these functions for additional smaller versions (Kast et al. 2015).

The yeast and filamentous fungi are host to self-replicating dsRNA agents located in the cytoplasm (Wickner 1986; Frank & Wolfe 2009). These RNA elements range in size from 1.5 kilobase-pairs to over 76 kbp. Genes from dsRNA elements in these fungi have transported to the nucleus and converted to DNA, where they were identified in the chromosomes (Frank & Wolfe 2009). Moreover, these elements have acquired genes from other organisms and other dsRNA elements through RNA-RNA recombination, making it possible for them to acquire sequences directly from exo-dsRNAs (Ramírez et al. 2017).

Presumptive exclusion of dsRNAs from the nucleus does not prevent interaction with these cytoplasmic genes. Neither the EPA staff nor the Decision-Making Committee addressed the broader diversity of genes or other genetic materials in eukaryotes.

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dsRNA is heritable

dsRNA molecules themselves can be amplified by RdRP acting on the target messenger RNA. Staff viewed this as a self-limiting reaction (paragraph 2.14 of Ref EPA 2018b). The description of the process by staff was based on the assumption that the only source of renewal of both the primary siRNA and secondary siRNAs is from primed RdRP activity. However, RdRP has an unprimed activity as well and this mechanism can generate secondary siRNA (Maida & Masutomi 2011). Further, the staff have erroneously categorized all secondary siRNAs as having 5´ triphosphates. It is only RNA molecules synthesized by unprimed synthesis that have 5´ triphosphates, and then will have them only in the 5´ most terminal siRNA molecules after Dicer cleavage (Maida & Masutomi 2011). Moreover, the staff statement is at odds with the ability of primary exo-dsRNA to generate secondary siRNAs that act on other genes (Simmer et al. 2010). Finally, it ignores the contribution that secondary siRNAs generated from exo-siRNAs make to transcriptional gene silencing and perpetuation of the effect, and off-target silencing, through interactions in the nucleus (Zhou et al. 2014).

Returning to the central point which is that while RNAi can be self-limiting (Houri-Zeevi & Rechavi 2017), it does not in all cases self-extinguish. It has been shown to transmit usually for around 3-5 generations, but has been observed to transmit for up to 80 generations (Houri-Zeevi & Rechavi 2017). Secondary small RNAs can prime tertiary small RNAs in the germline cells of the nematode *Caenorhabditis elegans* "and therefore set in motion a feed-forward process that could theoretically preserve transgenerational inheritance ad infinitum" (Rechavi & Lev 2017).

Critically, where transgenerational effects of exo-dsRNA have been studied at all, there is evidence that the self-limiting behavior of RNAi can be an active process (Houri-Ze'evi et al. 2016), not the outcome of dilution as hypothesized in the evidence relied upon by the EPA staff (paragraph 2.6 of Ref EPA 2018b). This could mean that there are other eukaryotic organisms in the vast repository native to New Zealand that lack this second tier of biochemistry modulating the response, or natural mutants that lack it. Interestingly, mutations in these limiting pathways in *C. elegans* cause hypersensitivity to exo-dsRNA stimulation (Houri-Zeevi & Rechavi 2017).

The limiting mechanisms are also not assurances that the transience of the effect is shorter than necessary to prevent a harmful effect of the treatment, should there be one. Moreover, the limiting response can be reduced by repeat exposures to the exo-dsRNA (Houri-Zeevi & Rechavi 2017). Repeat exposures are possible under the EPA Decision. According to the HSNO Act, an organism is modified when its genes or

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other genetic material have been modified, *not only after the modified genes or other genetic material are transmitted to offspring.* This is important to consider in particular for long-lived genetic resources or other species of conservation value, such as trees.

Unintended heritable changes

The common biochemistry accessed by exo-dsRNA and endo-dsRNA creates competition between them (Waldron 2016). Traits made stable and heritable by endo-dsRNA may be destabilized through competition with exo-dsRNA. If the outcome of the competition for Argonaute or other proteins is an alternative heritable pattern of gene expression, then this too is a heritable effect of treatment with exo-dsRNA.

Exposing the eukaryote *C. elegans* to exo-dsRNA downregulated the production of endo-dsRNAs that are necessary for the inheritance of endo-dsRNA effects (Houri-Ze'evi et al. 2016). This effect was not specific to the sequence of the genes controlled by particular endo-dsRNA, but to production of proteins necessary for intergenerational transmission of RNAi caused by endo-dsRNAs.

A critical feature of this observation is that any attempt to determine the longevity of exo-dsRNA-mediated RNAi must define how often an organism will be exposed to exo-dsRNA. This is because the "'transgenerational timer' is being reset by initiation of new RNAi responses, and therefore 'second triggers' extend the inheritance of ancestral silencing" (Houri-Ze'evi et al. 2016). Exposure frequencies will determine the duration of the effect both in time and number of generations.

Conclusion

The EPA Decision defines the use of dsRNA applied externally to eukaryotes as out of scope of their legislation. The Decision has important implications because all native and endogenous eukaryotes, even those yet to be discovered, as well as those described as exotics, with the exception of organisms banned by biosecurity laws, come under the jurisdiction of the HSNO Act.

A significant concern is that the Decision did not consider the *in vitro* techniques that could be used either to create, isolate or amplify the dsRNA. The Committee:

- put no constraints on the size of the dsRNA molecules.
- constrained treatment to organisms that are not excluded by the Biosecurity Act, *but did not constrain the source of the dsRNA to be used*.

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- removed any obligation to notify the use of *in vitro* conversion or synthesis of RNA genomes into dsRNA molecules.
- did not describe what it meant by external treatments, leaving chemical and biological vectors (Kolliopoulou et al. 2017) of any description possible.

Heritability

The EPA was certain that exo-dsRNA molecules could not be inherited by eukaryotes and this was the primary rationale for the determination that eukaryotes treated with them were not new or genetically modified organisms for the purposes of the HSNO Act. Prohibiting inheritance were various biological barriers (Fig. 1):

- exo-dsRNA does not mix with material in the nucleus of the cell. This, however, was shown to be false. Moreover, the EPA failed to account for replicating RNA elements in the cytoplasm of some eukaryotes, and the literature on RNA-RNA recombination.
- exo-siRNA is not reverse transcribed. This was shown to be plausible for some dsRNA molecules but demonstrably false for others.
- exo-dsRNA is not inheritable because it does not modify the DNA genome. This was shown to be false. First, exo-dsRNA may replicate independently of the DNA genome using RdRP-based amplification, as can other RNA-based elements in eukaryotes that are clearly genetic material. Second, exo-dsRNAs can modify DNA in chromosomes in some cell types or species. Modifications include heritable methylation of nucleotides and histones, DNA deletions and rearrangements, and changes in chromosome copy number.

In contrast to the EPA, the industry developing dsRNA treatments for broad scale environmental applications is convinced that the treatments result in heritable changes. For example, an exo-dsRNA treatment was used to effect a color change in flowers of petunia plants that produced progeny that retained the modified trait. Those progeny were used to illustrate the multi-generational claim of ownership made by the patent holder (see paragraph 0173 of Ref. Huang et al. 2018).

Terminology

The common understandings of terms not already defined in the HSNO Act served in this instance to reinforce the conclusion that dsRNA did not modify genes or other genetic material (EPA 2018b). For the meaning of genes and other genetic material, definitions were taken from the Oxford English Dictionary. A dictionary provides for its broad audience by supplying definitions that are useful for most applications readers may have, but are not technically comprehensive. For example, the

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dictionary definition is useful to say that chromosomes are genes and genetic materials, but experts do not turn to the dictionary to generate lists of all non-chromosomal genetic materials.

Reasonable sources for definitions on technical terms of central importance can include relevant international agreements in the area of biosafety, agriculture and conservation. These are also of practical value because they underpin international rules of trade and protection of organisms and biological material. In carefully negotiated and legally binding international instruments, it can be as deliberate to choose to not define particular terms as it is to define others. In the agreements described earlier, genetic material is not defined as specifically and exclusively the DNA of chromosomes in the nucleus of cells. Using the definitions from those instruments, modification of genetic material can result from changing the DNA of chromosomes in the nucleus, but also in other ways, such as by changing the replicating RNA elements in the cytoplasm of cells that have these, or the histone proteins of chromosomes in cells that will pass on an associated trait.

In Decision paragraph 4.9 the Committee said that it required evidence of dsRNA integrating into the genome (ie according to Decision paragraph 4.6, to be chemically attached to the DNA of chromosomes in the nucleus), or the dsRNA itself had to in some other way become inheritable, for the conclusion to be reevaluated. Implicit in the Decision text was that the modification had to be the continued propagation of the dsRNA, rather than the changes it made to the genetic material of an organism. Certainly if the dsRNA were propagated that would satisfy international definitions of modification, which also can include the change to the primary order of nucleotides in a DNA molecule as would result from linkage to a dsRNA molecule, if that could occur. However, the terms used by international instruments are also consistent with what agencies such as the UN Food and Agriculture Organization include, such as the "*chemical* modifications of DNA and chromatin, for instance, affecting the degree of chromatin compaction or the accessibility of regulatory sequences to transcription factors" (emphasis added to Ref CGRFA 2015). As discussed above, that is a kind of modification that can result from a treatment with exo-dsRNAs without reliance on continued transcription (Rechavi 2014). dsRNA can cause heritable effects without needing to propagate along with the modifications that it makes.

Other options

The EPA had other options. One would have been to decide for various reasons (eg that RNA was genetic material in its own right as in some viruses, or was a nucleic acid as referred to by the Protocol, or that the EPA had insufficient information about the diversity of eukaryotic responses to dsRNA to extrapolate further) that

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eukaryotes treated with dsRNA would be regarded as new organisms unless further information were to come to light to show the opposite. Specifically, EPA could require further evidence that molecules derived from dsRNA molecules *cannot* modify genes or other genetic material or *cannot* otherwise be passed to progeny of eukaryotic cells or organisms treated with externally applied dsRNA.

Had the EPA decided that eukaryotes treated with dsRNA were, at least for now, new or genetically modified organisms, it could have completed a risk assessment with the outcome possibly being that cells and organisms treated with external dsRNA in the laboratory were low risk, requiring the minimum biocontainment infrastructure. EPA could have decided this for the whole country, not requiring applications for further risk assessments and thus minimized costs to researchers and developers.

Alternatively, EPA could have extended approval to eukaryotic organisms held in a variety of containment facilities, tying the approval to physical containment conditions appropriate to the type of organism. Such facilities and requirements are already commonplace because of work with recombinant DNA.

Likewise, EPA could have reduced compliance costs for those using exo-dsRNA in contained facilities by limiting the approval to synthetically produced and short dsRNA molecules, as requested in the original application, prohibiting dsRNA derived from pathogens such as RNA viruses. Work using dsRNA derived from viruses would then require additional risk assessment.

The EPA decision was based on hypothetical barriers to inheritance that are not present in all eukaryotes. Furthermore, it makes possible the use of *in vitro* techniques that until recently were confined to the laboratory, making it possible to evaluate resulting genetically modified organisms before release. The topical dsRNA and other similar technologies intended to be used in the open air would instead allow the techniques of genetic engineering to be applied in the environment with no potential to evaluate the resulting products before release. In parallel with the use of "e" as a prefix for the adjective environment/al, I call this and other techniques of this type eGE, for environmental genetic engineering.

It is important to emphasize that RNA effects are still rapidly being described even in model research organisms. "Among some animal groups [in New Zealand], new species are being discovered faster than scientists can cope with them" (Manatū Taonga Ministry for Culture and Heritage) much less test them for dsRNA responses. The clear statements that there is likely to be much more to discover about dsRNA effects as more species are studied, statements made in the references used to develop advice from staff (EPA 2018b), were not mentioned in the advice provided

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to the Committee. The narrow treatment by EPA of how dsRNA could modify genes or genetic material is surprising given the nation's pride in its native biodiversity.

In the future, it might be determined that some or all uses of externally applied dsRNA create no unmanageable risks to human health, the environment, or to society. This would be a welcome finding because there is potential for dsRNA-based products to be at least short-term remedies for some problems. Coming to this position should be an evidence-based and precautionary process. Only that kind of process has the ability to build trust in responsible providers of biotechnology and agencies that serve to protect the public's interest in the environment. Taking shortcuts will inevitably invite delays.

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PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27545v1 | CC BY 4.0 Open Access | rec: 20 Feb 2019, publ: 20 Feb 2019

References

1996. Hazardous Substances and New Organisms Act. New Zealand.

Agrawal N, Dasaradhi PVN, Mohmmed A, Malhorta P, Bhatnagar RK, Mukherjee SK 2003. RNA interference: biology, mechanism, and applications. Microbiol. Mol. Biol. Rev. 67: 657-685.

Arhancet JPH, Summers N, Huang H; Apse, Llc, assignee 2016. Compositions and methods using capsids resistant to hydrolases.

Ashley P, Suanur C, Nicholas DL, Nadim M, Susan G 2017. Alternative pathways for Escherichia coli biofilm formation revealed by sRNA overproduction. Mol Microbiol 105(2): 309-325.

Bioland 2018. Chemically-modified siRNA. Retrieved 7 June 2018 http://bioland-sci.com/index.php?main_page=index&cPath=128_136

Brevik K, Lindström L, McKay SD, Chen YH 2018. Transgenerational effects of insecticides—implications for rapid pest evolution in agroecosystems. Curr. Opin. Insect Sci 26: 34-40.

Carthew RW, Sontheimer EJ 2009. Origins and mechanisms of miRNAs and siRNAs. Cell 136: 642-655.

CBD The Convention on Biological Diversity. Retrieved 26 June 2018 https://www.cbd.int/convention/

CBD Cartagena Protocol on Biosafety. Retrieved 19 April 2016 https://bch.cbd.int/protocol

CGRFA 2015. The Second Report on the World's Animal genetic resources for food and agricuture.

Crawford MJ, Li X, Kapoor M, Williams DJ; Monsanto Technology LLC, assignee 2014. Methods and compositions for plant pest control patent US 2014/0215656A1.

Dar SA, Thakur A, Qureshi A, Kumar M 2016. siRNAmod: A database of experimentally validated chemically modified siRNAs. Sci Rep 6: 20031.

Dard-Dascot C, Naquin D, d'Aubenton-Carafa Y, Alix K, Thermes C, van Dijk E 2018. Systematic comparison of small RNA library preparation protocols for next-generation sequencing. BMC Genom 19(1): 118.

de Parseval N, Lazar V, Casella J-E, Benit L, Heidmann T 2003. Survey of human genes of retroviral origin: identification and transcriptome of the genes with coding capacity for complete envelope proteins. J. Virol. 77: 10414-10422.

Deikman J, Schwartz SH, Zheng W, Gabriels SHEJ, Hresko MC, Li X, Tao N, Williams DJ, Xiong H; Monsanto Technology LLC, assignee 2017. Methods and compositions for delaying senescence and improving disease tolerance and yield in plants patent US 9,840,715 Bl.

Djupedal I, Ekwall K 2009. Epigenetics: heterochromatin meets RNAi. Cell Res 19: 282-295.

EPA 2018a. Decision.

EPA 2018b. EPA staff report. Determining whether eukaryotic cell lines treated with double-stranded RNA are genetically modified organisms.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27545v1 | CC BY 4.0 Open Access | rec: 20 Feb 2019, publ: 20 Feb 2019

696 Europa Genetic Resources. Retrieved 1 June 2018, updated 1 June 2018:
697 https://biodiversity.europa.eu/topics/genetic-resources
698 FAO Genetic resources. Retrieved 1 June 2018, updated 1 June 2018:
699 http://www.fao.org/genetic-resources/en/
700 FIFRA 2014. RNAi Technology as a Pesticide: Program Formulation for Human
701 Health and Ecological Risk Assessment.
702 Fillatti J, Froman B, Garvey GS, Hemmes JC; Monsanto Technology LLC, assignee
703 2012. Method for improving shelf life by regulating expression of polyphenol
704 oxidase.
705 Frank AC, Wolfe KH 2009. Evolutionary capture of viral and plasmid DNA by yeast
706 nuclear chromosomes. Eukaryot Cell 8: 1521-1531.
707 Frech B, Peterhans E 1994. RT-PCR: 'background priming' during reverse
708 transcription. Nucl. Acids Res 22: 4342-4343.
709 Ghildiyal M, Zamore PD 2009. Small silencing RNAs: an expanding universe. Nat Rev.
710 Genet. 10: 94-108.
711 Gibbs MJ, Weiller GF 1999. Evidence that a plant virus switched hosts to infect a
712 vertebrate and then recombined with a vertebrate-infecting virus. Proc. Natl.
713 Acad. Sci. USA 96: 8022-8027.
714 Goic B, Vodovar N, Mondotte JA, Monot C, Frangeul L, Blanc H, Gausson V, Vera-
715 Otarola J, Cristofari G, Saleh M-C 2013. RNA-mediated interference and
716 reverse transcription control the persistence of RNA viruses in the insect
717 model Drosophila. Nat. Immunol 14: 396.
718 Gorlich D, Kutay U 1999. Transport between the cell nucleus and the cytoplasm.
719 Annu. Rev Cell Dev. Biol. 15: 607-660.
720 Guang S, Bochner AF, Pavelec DM, Burkhart KB, Harding S, Lachowiec J, Kennedy S
721 2008. An argonaute transports siRNAs from the cytoplasm to the nucleus.
722 Science 321(5888): 537-541.
723 Gupta A, Pal RK, M.V. R 2013. Delayed ripening and improved fruit processing
724 quality in tomato by RNAi-mediated silencing of three homologs of 1-
725 aminopropane-1-carboxylate synthase gene. J Pl Physiol 170: 987-995.
726 Han R, Xu L, Wang T, Liu B, Wang L 2017. A small regulatory RNA contributes to the
727 preferential colonization of Escherichia coli O157:H7 in the large intestine in
728 response to a low DNA concentration. Front Microbiol 8: 274.
729 Hatch E, Hetzer M 2014. Breaching the nuclear envelope in development and
730 disease. J Cell Biol 205(2): 133-141.
731 Heinemann JA, Agapito-Tenfen SZ, Carman JA 2013. A comparative evaluation of the
732 regulation of GM crops or products containing dsRNA and suggested
733 improvements to risk assessments. Environ Int 55: 43-55.
734 Holliday R, Grigg GW 1993. DNA methylation and mutation. Mut Res 285: 61-67.
735 Houri-Ze'evi L, Korem Y, Sheftel H, Faigenbloom L, Toker IA, Dagan Y, Awad L,
736 Degani L, Alon U, Rechavi O 2016. A tunable mechanism determines the
737 duration of the transgenerational small RNA inheritance in C. elegans. Cell
738 165(1): 88-99.
739 Houri-Zeevi L, Rechavi O 2017. A matter of time: small RNAs regulate the duration of
740 epigenetic inheritance. Trends Genet 33(1): 46-57.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27545v1 | CC BY 4.0 Open Access | rec: 20 Feb 2019, publ: 20 Feb 2019

741 Huang S, Iandolino AB, Peel GJ; Monsanto Technology LLC, assignee 2018. Methods
742 and compositions for introducing nucleic acids into plants. United States
743 patent US 2018/0163219 Al.
744 ITPGRFA International Treaty on Plant Genetic Resources in Food and Agriculture.
745 Kalinava N, Ni JZ, Gajic Z, Kim M, Ushakov H, Gu SG 2018. C. elegans heterochromatin
746 factor SET-32 plays an essential role in transgenerational establishment of
747 nuclear RNAi-mediated epigenetic silencing. Cell Rep 25(8): 2273-2284.e3.
748 Kalluri R, Kanasaki K 2008. Generic block on angiogenesis. Nature 452: 543.
749 Kast A, Voges R, Schroth M, Schaffrath R, Klassen R, Meinhardt F 2015. Autoselection
750 of cytoplasmic yeast virus Like elements encoding toxin/antitoxin systems
751 involves a nuclear barrier for immunity gene expression. PLoS Genetics
752 11(5).
753 Kershen DL 2015. Sustainability Council of New Zealand Trust v the Environmental
754 Protection Authority: gene editing technologies and the law. GM Crops Food.
755 Khurana JS, Clay DM, Moreira S, Wang X, Landweber LF 2018. Small RNA-mediated
756 regulation of DNA dosage in the ciliate Oxytricha. RNA 24(1): 18-29.
757 Killmer JL, Kumar A, Mclaughlin PD, Arhancet JPH; Apse, Inc., assignee 2016.
758 Methods and compositions for controlling ants.
759 Kim VN, Jinju H, Siomi MC 2009. Biogenesis of small RNAs in animals. Nat Rev. Mol.
760 Cell Biol. 10(2): 126-139.
761 Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, Baffi JZ,
762 Albuquerque RJC, Yamasaki S, Itaya M, Pan Y and others 2008. Sequence- and
763 target-independent angiogenesis suppression by siRNA via TLR3. Nature
764 452: 591.
765 Kolliopoulou A, Taning CNT, Smagghe G, Swevers L 2017. Viral delivery of dsRNA for
766 control of insect agricultural pests and vectors of human disease: prospects
767 and challenges. Front Physiol 8.
768 Lam E; 2012. Edible transgenic plants as oral delivery vehicles for RNA-based
769 therapeutics patent WO 2012135820 A2.
770 Li L-C 2008. The multifaceted small RNAs. RNA Biol. 5: 61-64.
771 Liu S, da Cunha AP, Rezende RM, Cialic R, Wei Z, Bry L, Comstock LE, Gandhi R,
772 Weiner HL 2016. The host shapes the gut microbiota via fecal microRNA. Cell
773 Host Microbe 19(1): 32-43.
774 Maida Y, Masutomi K 2011. RNA-dependent RNA polymerases in RNA silencing. Biol.
775 Chem. 392: 299-304.
776 Manatū Taonga Ministry for Culture and Heritage Te Ara - Encyclopedia of New
777 Zealand.
778 Mao H, Zhu C, Zong D, Weng C, Yang X, Huang H, Liu D, Feng X, Guang S 2015. The
779 Nrde pathway mediates small-RNA-directed histone H3 lysine 27
780 trimethylation in Caenorhabditis elegans. Curr Biol 25(18): 2398-2403.
781 Marraffini LA, Sontheimer EJ 2010. CRISPR interference: RNA-directed adaptive
782 immunity in bacteria and archaea. Nat Rev. Genet. 11: 181-190.
783 Mars RAT, Nicolas P, Denham EL, van Dijl JM 2016. Regulatory RNAs in Bacillus
784 subtilis: a Gram-Positive perspective on bacterial RNA-Mediated regulation
785 of gene expression. Microbiol Mol. Biol Rev 80(4): 1029-1057.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27545v1 | CC BY 4.0 Open Access | rec: 20 Feb 2019, publ: 20 Feb 2019

786 Massirer KB, Pasquinelli AE 2013. MicroRNAs that interfere with RNAi. Worm 2(1):
787 e21835.
788 Matzke MA, Birchler JA 2005. RNAi-mediated pathways in the nucleus. Nat. Rev.
789 Genet. 6: 24-35.
790 Mochizuki K, Gorovsky MA 2004. Small RNAs in genome rearrangement in
791 Tetrahymena. Curr. Opin. Genet. Develop. 14: 181-187.
792 MPI Biosecurity. Retrieved 26 June 2018 https://www.mpi.govt.nz/law-and-
793 policy/legal-overviews/biosecurity/
794 MPI 2018. Declaration for potential risk goods in catalogues. Retrieved 7 June 2018
795 https://www.mpi.govt.nz/dmsdocument/5134/send
796 Ngo KA, Jones SA, Church TM, Fuschino ME, George KS, Lamson DM, Maffei J, Kramer
797 LD, Ciota AT 2017. Unreliable inactivation of viruses by commonly used lysis
798 buffers. Appl Biosaf 22(2): 56-59.
799 Noto T, Mochizuki K 2017. Whats, hows and whys of programmed DNA elimination
800 in Tetrahymena. Open Biology 7(10).
801 Nowacki M, Haye JE, Fang W, Vijayan V, Landweber LF 2010. RNA-mediated
802 epigenetic regulation of DNA copy number. Proc. Natl. Acad. Sci. USA
803 107(51): 22140.
804 Nowacki M, Vijayan V, Zhou Y, Schotanus K, Doak TG, Landweber LF 2008. RNA-
805 mediated epigenetic programming of a genome-rearrangement pathway.
806 Nature 451(7175): 153-8.
807 Numata K, Ohtani M, Yoshizumi T, Demura T, Kodama Y 2014. Local gene silencing
808 in plants via synthetic dsRNA and carrier peptide. Pl Biotechnol. J 12(8):
809 1027-1034.
810 Papenfort K, Vanderpool CK 2015. Target activation by regulatory RNAs in bacteria.
811 FEMS Microbiol. Rev 39: 362-378.
812 Ramírez M, Velázquez R, López-Piñeiro A, Naranjo B, Roig F, Llorens C 2017. New
813 insights into the genome organization of yeast killer viruses based on
814 “atypical” killer strains characterized by high-throughput sequencing. Toxins
815 9(9).
816 Rankin CH 2015. A review of transgenerational epigenetics for RNAi, longevity,
817 germline maintenance and olfactory imprinting in Caenorhabditis elegans. J.
818 Exp. Biol. 218(1): 41-49.
819 Rechavi O 2014. Guest list or black list: heritable small RNAs as immunogenic
820 memories. Trends Cell Biol 24(4): 212-220.
821 Rechavi O, Lev I 2017. Principles of transgenerational small RNA inheritance in
822 Caenorhabditis elegans. Curr. Biol 27(14): R720-R730.
823 Sammons RD, Ivashuta S, Liu H, Wang D, Feng PCC, Kouranov AY, Andersen SE;
824 Monsanto Technology LLC, assignee 2011. Method for controlling herbicide-
825 resistant plants patent US9121022B2.
826 Sharples FE 2017. Synthesis of Horsepox from mail-order DNA. Appl Biosaf 22: 90-
827 91.
828 Sigma 2018. Custom Options and Services. Retrieved 7 June 2018
829 https://www.sigmaaldrich.com/life-science/functional-genomics-and-
830 rnai/sirna/learning-center/mission-sup-reg0/custom-options-and.html

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831 Silas S, Mohr G, Sidote DJ, Markham LM, Sanchez-Amat A, Bhaya D, Lambowitz AM,
832 Fire AZ 2016. Direct CRISPR spacer acquisition from RNA by a natural
833 reverse transcriptase–Cas1 fusion protein. Science 351(6276).
834 Simmer F, Buscaino A, Kos-Braun IC, Kagansky A, Boukaba A, Urano T, Kerr ARW,
835 Allshire RC 2010. Hairpin RNA induces secondary siRNAs synthesis and
836 silencing in trans in fission yeast. EMBO Rep 11(2): 112-8.
837 siRNAmod 2018. A database of experimentally validated chemically modified
838 siRNAs. Retrieved 7 June 2018
839 http://crdd.osdd.net/servers/sirnamod/index.php
840 Sjöström AE, Sandblad L, Uhlin BE, Wai SN 2015. Membrane vesicle-mediated
841 release of bacterial RNA. Sci Rep 5: 15329.
842 Smoyer CJ, Jaspersen SL 2014. Breaking down the wall: the nuclear envelope during
843 mitosis. Curr. Opin. Cell Biol. 26: 1-9.
844 Trought K 2018. APP203395.
845 Van BE, Kubler L, Raemaekers R, Bogaert T, Plaetinck G; Devgen NV assignee 2011.
846 Methods for controlling pests using RNAi patent EP2347759A2.
847 Various Worm Book.
848 Waldron D 2016. Small RNAs: regulating transgenerational epigenetics. Nature 17:
849 315.
850 Whyard S, Cameron FH, Moghaddam M, Lockett TJ; Commonwealth Scientific and
851 Industrial Research Organization, assignee 2011. Delivery of dsRNA to
852 arthropods. Europe patent EP 2 333 061 A1.
853 Wickner RB 1986. Double-stranded RNA replication in yeast: the killer system. Annu
854 Rev Biochem 55: 373-395.
855 Wickner RB, Edskes HK 2015. Yeast killer elements hold their hosts hostage. PLoS
856 Genet 11(5): e1005139.
857 Woodhouse RM, Buchmann G, Hoe M, Harney DJ, Low JKK, Larance M, Boag PR, Ashe
858 A 2018. Chromatin modifiers SET-25 and SET-32 are required for
859 establishment but not long-term maintenance of transgenerational
860 epigenetic inheritance. Cell Rep. 25(8): 2259-2272.e5.
861 Zhou X, Xu F, Mao H, Ji J, Yin M, Feng X, Guang S 2014. Nuclear RNAi contributes to
862 the silencing of off-target genes and repetitive sequences in Caenorhabditis
863 elegans. Genetics 197(1): 121-32.
864 Zilberman D 2017. An evolutionary case for functional gene body methylation in
865 plants and animals. Genome Biol. 18(1): 87.
866
867

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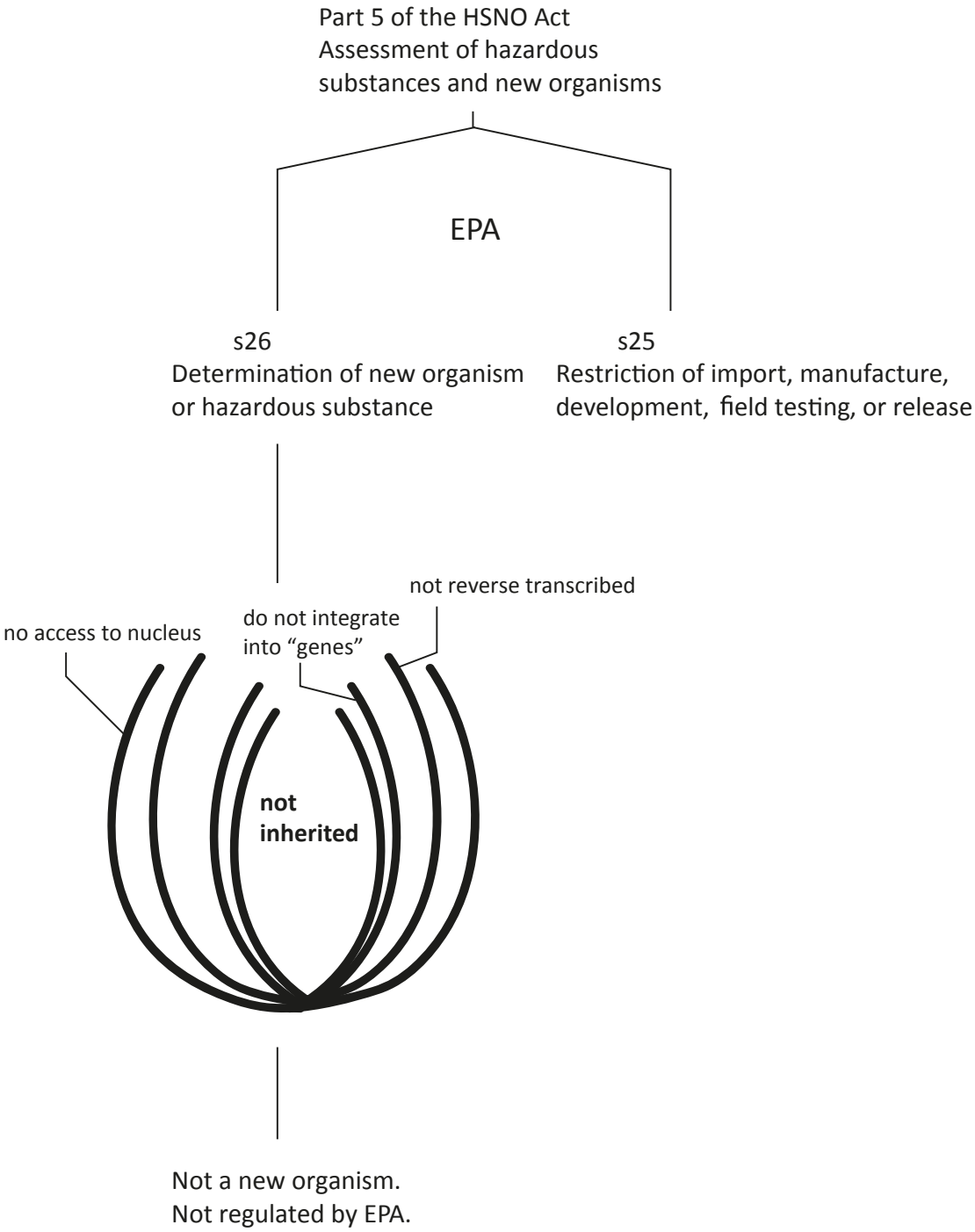

Figure 1: Context of the EPA Decision. The EPA has two different activities described by sections 25 and 26 of the HSNO Act. (Left) Under s26, EPA decided that eukaryotes treated with exo-dsRNA were not new or genetically modified organisms because exo-dsRNA is not inheritable. That conclusion is pictured as the center of an onion (center left), further protected by several additional layers that all contribute to increasing certainty in the conclusion. The layers are, from outermost, that exo-

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dsRNA: has no access to the nucleus and genes or other genetic material therein, cannot be reverse transcribed into DNA, and therefore cannot modify genes or other genetic material in the nucleus through integration, and it is not the genes or other genetic material of a eukaryote. (Right) If EPA decided that treatment of eukaryotic cells or organisms with dsRNA modified genes or genetic material by *in vitro* techniques, then s25 would apply.

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Table 1. Common siRNA *in vitro* chemical modifications

2´ O-Methyl phosphoramidites	2´-O-Me-rA, 2´-O-Me-rC, 2´-O-Me-rG, 2´-O-Me-rU	Increase stability, longer lasting RNAi effects
2´ Fluoro phosphoramidites	2´-FluoC, 2´-FluoU	
5´ modifications	5´-Amino, 5´-Biotin, 5´-Cholesterol, 5´-Phophorylation and 5´-Thio	Various reasons, e.g. cholesterol for improved penetration through membranes.
3´ modification	3´-amino	
Table content amalgamated from several sources (Refs Dar et al. 2016; Bioland 2018; Sigma 2018).		

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