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Expert scientific opinion on the status of certain new techniques of genetic modification under Directive 2001/18/EC

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Common Abbreviations

DNA	deoxyribonucleic acid
COGEM	Netherlands Commission on Genetic Modification
GMAG	Genetic Manipulation Advisory Group
GMO	genetically modified organism
LMO	living (genetically) modified organism
NIH	National Institutes of Health
OGE	oligonucleotide-directed genetic engineering
ODM/OMM	oligonucleotide-direct mutagenesis/ oligonucleotide-mediated mutagenesis
rDNA	recombinant DNA
Recombineering	*in vivo* genetic engineering with short DNA molecules
RNA	ribonucleic acid

Overview

Genetic technologies change. Are new techniques resulting in organisms that should be regulated by member states of the European Union (Podevin et al., 2012)? Organisms considered to be genetically modified cannot be released into the environment before, among other things, a scientific risk assessment has been completed. EU Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms describes in general terms what is and what is not a genetically modified organism (GMO) (Spranger, 2015). Those GMOs in the scope of the Directive are subjected to a mandatory risk assessment as set out in Articles 2(8), 4 and 13. A decision-maker takes the findings of the risk assessment into account, may impose additional risk management requirements, and also takes into account ethical considerations[1].

The GMO regulations were established in response to the new technologies emerging in the 1970s. These regulations have evolved over time and jurisdiction. Between 1990 and 2001 Directive 90/220/EEC on the deliberate release into the environment of genetically modified organisms was updated to EU Directive 2001/18/EC in part to better capture the scope of coverage. The question here is whether variations of certain techniques, that just in the last few years are beginning to create potential products for release into the environment, are subject to the 2001 Directive.

The two general conclusions of the analysis detailed in this report are:

1. application of the techniques discussed creates genetically modified organisms in the sense of Article 2 of Directive 2001/18/EC; and
2. the techniques do not pass at least three tests needed for them to be excluded from the scope of Directive 2001/18/EC. They fail because —
    a. they are not well established as techniques with a long safety record (in general terms, as opposed to safe/unsafe single products);
    b. they are not the techniques referred to in Article 3 and listed in Annex 1 B of Directive 2001/18/EC because they are not 'mutagenesis' in the same scientific way that the listed excluded techniques are mutagenesis; and
    c. they are techniques based on the use of recombinant nucleic acid molecules.

I have found that the new techniques have substantive scientific continuity with earlier variations of regulated techniques and have no properties that are

[1] "Member States may take into consideration ethical aspects when GMOs are deliberately released or placed on the market as or in products" (paragraph 9 of Directive 2001/18/EC). This theme also appears in Regulation (EC) No. 1829/2003 on genetically modified food and feed Article 7(1) with reference to "other legitimate factors" and in Council Conclusions of December 2008 (16882/08): cf. (ii): "under Directive 2001/18/EC, the Commission is to submit a specific report on the implementation of the Directive, including an assessment, inter alia, of socio-economic implications of deliberate releases and placing on the market of GMO."

outside of defining features of regulated techniques at the time that Directive 2001/18/EC was written.

Many of the new techniques involve small polymers of nucleic acids called oligonucleotides.[2] These nucleic acid molecules are introduced into cells[3] where they interact with, among other things, a target nucleic acid of (partial to completely) complementary sequence. That interaction results in changes to the target sequence and, consequently, the use of oligonucleotides in this way has been likened to 'mutagenesis'. The techniques using oligonucleotides are commonly called, among other names, oligonucleotide-directed mutagenesis (ODM), chimeraplasty, oligonucleotide-mediated gene modification (OGM) recombineering (recombinogenic engineering), genome editing, genomic engineering, site-directed mutagenesis (SDM) or oligonucleotide-mediated mutagenesis (OMM).

There can be many terms used in science that have a specific or technical meaning. Despite this, as the proliferation of aliases of ODM illustrates, there can be many synonymous uses of terms even while others hold fastidiously to non-overlapping definitions. Unfortunately, that is the case not just for what is called ODM, but for many of the key terms used in Directive 2001/18/EC. It would be a mistake to ignore common homonymous meanings or synonymous uses of these terms when attempting to interpret Directive 2001/18/EC (Appendix One).

Mutagenesis or genetic engineering?

The European Commission New Techniques Working Group (EC, 2012) defined ODM as follows:

Oligonucleotide directed mutagenesis (ODM) employs oligonucleotides for targeted (site-specific) induction of point mutations[4].

Oligonucleotides of approximately 20 to 100 nucleotides are delivered to the cells by methods suitable for the different cell types (including electroporation, polyethylene-glycol-mediated transfection, natural uptake). ***The technique exploits the sequence specific interaction of the oligonucleotide with the resident DNA of the cells****, resulting in gene targeting. This directs the attempted genetic modification to a specific region in the DNA or even to a specific base pair. The genetic modification can be the induction of a point mutation or reversion of an existing mutation which may lead to changes in the expression of a gene (emphasis added to EC, 2012).*

The definition of ODM used by the New Techniques Working Group describes the essence of such 'new techniques', namely the sequence-specific interaction of nucleic acids, also called hybridization, *in vivo*.[5] Parameters, such as the number

[2] Chemically or enzymatically synthesized *in vitro.*

[3] Introduced into cells by a variety of physical and bio-derived methods none of which occur naturally by mating and/or natural recombination.

[4] *replacement of one or a few base pairs or introduction of short deletions*

[5] "Specific hybridization is a critical step for determining the efficiency of the gene-correction process" Gocal, G. (2014). Non-transgenic trait development in crop plants using oligo-directed mutagenesis: Cibus' Rapid Trait Development System. In NABC Report 26 New DNA-Editing Approaches Methods, Applications and Policy for Agriculture, A. Eaglesham, and R.W.F. Hardy, eds. (North American Agricultural Biotechnology Council Report), pp. 97-105.

of nucleotides and range of mutations, are useful guidelines but not exhaustive. I also consider, for example, the additional capacity to introduce insertions and deletions. The definition is consistent with the suite of techniques that are described in the literature as causing homology-dependent, recombination-mediated, genetic engineering (Sargent et al., 2011).

Descriptions of these techniques have frequently included that their purpose was to create genetically modified organisms through genetic engineering. "In the past few years, *in vivo* technologies have emerged that, due to their efficiency and simplicity, may one day replace standard genetic engineering techniques" (Court et al., 2002).

Some accounts of the genesis of homology-dependent, recombination-mediated genetic engineering suggest that the first applications date back to the late 1970s (Pauwels et al., 2014), with the term oligonucleotide-directed mutagenesis applied to modifications achieved *in vivo* appearing by 1986 (Rivera-Torres and Kmiec, 2015). *In vivo* homology-dependent, recombination-mediated genetic engineering was used to generate deletions at least by the 1990s in the yeast *Saccharomyces cerevisiae* (Baudin et al., 1993; Moerschell et al., 1988). This use resulted in organisms with new combinations of genetic material capable of continued propagation. Variations of this first description were adapted to more organisms ranging from the bacterium *Escherichia coli* to mice (Copeland et al., 2001; Ellis et al., 2001). Practical protocols that can be applied to a broader range of organisms appeared more recently (Pauwels et al., 2014).

Researchers who were developing these and related techniques often described their purpose as a form of genetic engineering. For example:

- *Genetic engineering has been instrumental in revolutionizing studies in molecular biology for over 30 years since the discovery of restriction enzymes.* Escherichia coli *has been the standard host used to recover the products of this* in vitro *genetic engineering. Since the late 1990s, however, new* in vivo *technologies have emerged that greatly simplify, accelerate, and expand genetic engineering in* E. coli, Salmonella enterica*, and other organisms. Now, within a week a researcher can modify any nucleotide(s) of choice in almost any manner. Further, these genetic engineering technologies do not rely on* in vitro *reactions carried out by restriction enzymes and DNA ligase. Instead, they utilize the bacteriophage λ homologous recombination proteins collectively called "Red" to directly modify DNA within a bacterial cell (emphasis added to Sawitzke et al., 2007).*
- *Genetic engineering can now be accomplished in* E. coli *without using restriction endonucleases and DNA ligase. RecET/λ Red homologous recombination can efficiently modify chromosomal or plasmid DNA in vivo by recombination with linear [single-stranded]DNA or [double-stranded]DNA electroporated into a cell. The most important aspects of recombineering are that only short homology segments are required to direct the recombination, and recombination efficiency rates allow recombinants to be screened rather than selected (Copeland et al., 2001).*
- *In contrast to classical genetic engineering techniques, recombineering does not require construction of plasmid or phage DNA intermediates containing*

the appropriately pre-engineered homology segments. All that is required in vitro *is the synthesis of standard oligonucleotides (oligos) that provide the homology. These oligos can be used directly for recombineering or for construction of PCR products that are used for recombineering. For effective gene replacements, the PCR products are generated with ~50-[base-pair] ends that are homologous to sequence targets in the genome (Court et al., 2002).*

- *For more than 50 years, those engineering genetic material have pursued increasingly challenging targets. During that time, the tools and resources available to the genetic engineer have grown to encompass new extremes of both scale and precision, opening up new opportunities in genome engineering (Carr and Church, 2009).*
- *In discussing the idea of genome engineering, we apply this working definition: extensive and intentional genetic modification of a replicating system for a specific purpose (Carr and Church, 2009).*
- *Oligonucleotide-mediated genome engineering is a practical method for performing site-directed mutagenesis to enable the rapid generation of various rationally designed organisms (DiCarlo et al., 2013).*

From publication of the first demonstrations of ODM, it has been described with clarity as a means to create genetically modified organisms. This seems to have been lost in many narratives that concentrate on the term mutagenesis.

What this report is focussed on is the new use of oligonucleotides in techniques that require the oligonucleotide to be taken up by a cell where it then interacts with a target molecule. Nevertheless, the broader history of the use of oligonucleotides is helpful for understanding them and what characteristics they possess that is relevant to the Directive. Therefore, developments in oligonucleotide use will be covered as required from approximately the 1970s till now.

Older uses of oligonucleotides were predominantly *in vitro* reactions, where they were incorporated into a DNA molecule by reactions of DNA replication or ligation, rather than homologous recombination (Botstein and Shortle, 1985; Shortle et al., 1981). What was introduced into cells tended to be much larger derivative molecules. Those techniques significantly pre-date the Directive and are within its coverage.

Naming the techniques described by the New Techniques Working Group 'mutagenesis', creates a link to the list of techniques in Annex 1 B of the Directive that describe techniques of genetic modification that are specifically excluded from coverage by the Directive. Is this appropriate? Most of this report is devoted to answering that question. The point I make here is that choosing to call these techniques 'mutagenesis' does not prove them to be less like the mutagenesis caused by the insertion of a transgene (included in the scope of the Directive), or more like the mutagenesis caused by a chemical mutagen or radiation source (excluded from the scope of the Directive) (Pauwels et al., 2014).

Indeed, any change to a DNA sequence can be described as a mutation.[6] Any process by which it is brought about can be described as mutagenesis. Thus, describing any technique that causes mutations as 'mutagenesis' appears to be an obvious thing to do (Rivera-Torres and Kmiec, 2015), but not useful for determining which techniques the Directive includes and which it excludes from coverage.

A helpful description of the evolution of techniques applied by people for the purpose of changing DNA sequence was provided by Segal and Meckler (Segal and Meckler, 2013). They organised techniques into three categories. 1. Random mutagenesis with screening are methods that "generally involve using radiation, chemicals such as ethylnitrosourea (ENU), or transposons to generate low levels of random mutations" (Segal and Meckler, 2013). 2. Biased targeting with selection are methods that "use many copies of an exogenous donor DNA molecule with an insertion cassette flanked by long (10–20-kb) regions of homology to the desired target site. The cell's homologous recombination (HR) DNA-repair mechanisms could then introduce the insertion cassette at the target site in a precise and predictable manner" (Segal and Meckler, 2013). It is also possible to conduct biased targeting using shorter oligonucleotides rather than large DNA fragments, as in ODM (EC, 2012; Segal and Meckler, 2013). 3. True targeting makes use of nucleases such as engineered homing endonucleases, ZFN, TALEN or Cas9 for "genome-engineering methods that can be categorized as true targeting and then screening individuals by phenotype or genotype for mutations that occurred at the desired location" (Segal and Meckler, 2013).

Techniques that involve the use of proteins that create sequence-directed double-strand breaks at target sequences in a genome (Pauwels et al., 2014) are outside of the terms of reference for this report. However, there also will be obvious implications for those techniques.

As the quotes above show, the use of oligonucleotides is intended to be a form of genetic engineering using tools that are a continuum of those used in other forms of genetic engineering (Lin and Qin, 2014). I therefore will be true to this terminology in the report using the term oligonucleotide-directed genetic engineering (OGE) rather than ODM.

The term oligonucleotide-directed genetic engineering (OGE) is adopted in this report as the preferred alias for techniques such as those defined by the New Techniques Working Group as ODM.

To determine if OGE is a technique that creates genetically modified organisms within the Directive's description of regulated GMOs, I have reviewed the scientific meaning of the relevant words and phrases in the Directive, and the history of the development of those techniques and the Directive.

I found that some key words in the Directive have multiple and inconsistent meaning in the scientific literature and did before 2001, when the Directive was written. It is not possible therefore to simply choose a meaning based on current

[6] "A gene mutation is a permanent alteration in the DNA sequence that makes up a gene…Mutations range in size; they can affect anywhere from a single DNA building block (base pair) to a large segment of a chromosome that includes multiple genes" http://ghr.nlm.nih.gov/handbook/mutationsanddisorders/genemutation.

usage. However, I found good evidence that meanings of these words and phrases in use prior to 2001 can help to clarify the Directive. Importantly, key phrases used by the Directive are essentially unchanged from guidelines written decades before. This observation establishes a regulatory convention that I believe clearly applies generally to both OGE and oligonucleotides.

Based on the history of the science contemporaneous with the development of regulation of genetically modified organisms, I conclude that OGE is described by the list of techniques within the coverage of, and not captured by the list of techniques exempted from, Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms.

Distinguishable and safe?

OGE can create organisms with genetic modifications indistinguishable from modifications caused by other means, notably means that are excluded from GMO legislation. The justification for excluding certain techniques includes that they were already well established and in wide use when the GMO regulations were made (Krämer, 2015; Lusser and Davies, 2013; Spranger, 2015). That combination of time and experience had established a 'long safety record'.[7]

According to some, if products created using OGE are indistinguishable from the products created by processes excluded from legislation, e.g. chemical and radiation mutagenesis, then both have an equal potential to cause harm and neither should be regulated (Breyer et al., 2009; Lusser and Davies, 2013; Pauwels et al., 2014).[8]

Distinguishability of products is neither relevant to the definition of a genetically modified organism nor the description of processes through which they can be made.

This reasoning is based upon distinguishability of products and not distinguishability of techniques, the latter being the focus of the Directive. Even setting this issue aside, the ability to distinguish between two products of different techniques is itself largely a function of existing technology.[9] As technology changes it might also be able to reveal currently invisible differences in the products of some

[7] "This Directive should not apply to organisms obtained through certain techniques of genetic modification which have conventionally been used in a number of applications and have a long safety record" (paragraph 17 of Directive 2001/18/EC).

[8] Annex 1 B does not state the criteria used to assemble the list of excluded techniques but the list is widely regarded as including techniques with a long safety record Krämer, L. (2015). Legal questions concerning new methods for changing the genetic conditions in plants. Test Biotech, Spranger, T.M. (2015). Legal analysis of the applicability of Directive 2001/18/EC on genome editing technologies. German Federal Agency for Nature Conservation. I am not arguing that techniques excluded by Directive 2001/18/EC always make safe products (see also footnote 29). Likewise, I don't presume that the Directive was written to serve as the instrument to regulate all kinds of living things. Instead, the Directive was written to be the instrument to regulate GMOs created by particular techniques. Those techniques were chosen on the basis of being different from techniques introduced prior to and independent from development of genetic engineering technology.

[9] Paragraph 54 of "Sustainability Council of New Zealand Trust v Environmental Protection Authority" CIV 2013-485-877[2014] NZHC 1067. https://forms.justice.govt.nz/search/Documents/pdf/jdo/39/alfresco/service/api/node/content/workspace/SpacesStore/68d3a84c-ce73-4034-ae61-db02e1419157/68d3a84c-ce73-4034-ae61-db02e1419157.pdf. Access date 23 October 2015.

techniques and those made by natural or exempted processes.[10] In which case, organisms deemed to not be legally 'new' now might be deemed to be legally new later and this would require retrospective risk assessments, creating commercial uncertainty and liability.

Importantly, the ability to distinguish between two organisms after the fact may not be relevant to the Directive. For example, through some techniques it is possible to create an organism that would be extremely unlikely to come into being without the use of regulated techniques (e.g. transgene insertion) and would be within the coverage of the Directive. However, just because it was unlikely to have come into being through natural processes does not mean that it could necessarily be distinguished from an organism created through exempted or natural processes. If someone happened upon such an organism not knowing its origin they could conjecture as to its artificial origin. However, one cannot examine its DNA and determine absolutely that it was artificially created without having available a parental organism that lacked this particular combination of genetic material.

By way of example, suppose I take three identical yeast cells. The first yeast cell undergoes a single natural mutation changing a single nucleotide within the genome. To the second, I apply the OGE technique using an oligonucleotide designed to alter the genome at exactly the same place as the natural mutation in the first cell resulting in the same change. To the third, I 'splice' DNA the same as the mutation in the first cell at the site of that mutation. The result is again three identical yeast cells. Each of these will be different from the original organisms in exactly the same way, and each will be completely indistinguishable from the others.

All three cells were able to be made prior to the existence of the Directive and thus 'indistinguishability' based on existing technology does not appear to be a criterion necessary for coverage by the Directive. In fact, it would not even now be possible to reliably detect, on a routine basis, a regulated genetic modification of a maize seed without the diagnostic information provided by a law abiding developer (Heinemann et al., 2004).

Moreover, technological distinguishability may not be sufficient to nullify ethical considerations that also fall within the coverage of the Directive.[11]

A corollary of this question is one of safety and consistency. Should technically indistinguishable products be considered equally safe and therefore regulated the same way? Referring to the yeast cells above, clearly it is possible to create through different processes technically indistinguishable products. However, this observation is not evidence that the techniques behind their creation are

[10] By way of example, inability to distinguish between people suspected of theft because of a lack of technology has not been used to define stealing as 'not a crime'. This is fortunate because changes in technology, e.g. fingerprinting technology, have progressively contributed to efforts to distinguish between suspects. The adoption of gloves by thieves eroded the ability to distinguish between some suspects, but did not threaten the definition of theft. A change in technology, the introduction of among other things DNA fingerprinting technology, again helped to distinguish between suspects.

[11] "Member States may take into consideration ethical aspects when GMOs are deliberately released or placed on the market as or in products" (paragraph 9 of Directive 2001/18/EC).

limited to creating the same range of potential products (Figure 1)[12]. The range of differences might expand in time, with changes in technology, as mentioned above.

Distinguishing between some products can be challenging, but this is beside the point. The regulations do not define their scope by the similarity or otherwise of products, but by the differences between techniques (scientifically, ethically and through history of use). The challenge of distinguishing between products could be addressed several ways. For example, the ability to identify products made using techniques within the coverage of the Directive could also be required of developers, just as diagnostic information necessary to distinguish, identify and track other GMOs is currently a requirement for release. This may also be desirable for developers who could use these methods to defend against unlicensed use of their intellectual property.

Herein the precautionary principle[13] is critical to consider. It is only possible to know that any particular product of a new technique, such as OGE, is identical to a product made through an exempted technique by subjecting it to a risk assessment as set out in Articles 2(8), 4 and 13 of Directive 2001/18/EC. It will only be subject to a risk assessment if it was developed by a technique that is

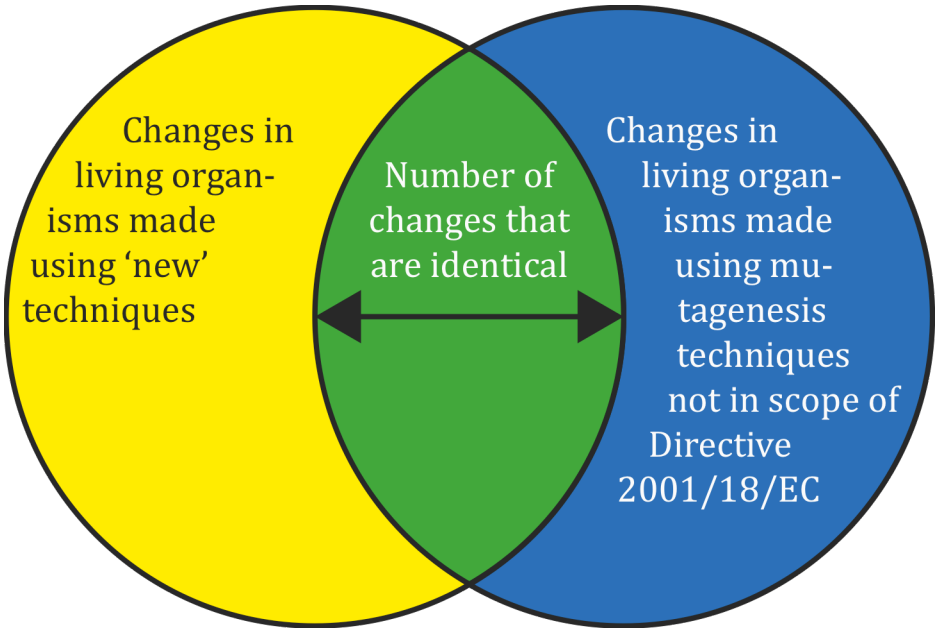

Figure 1. Venn diagram illustrating the sets of changes that are possible using different techniques. The 'green' overlap may describe a small or a large proportion of the changes in common between the sets, but that can vary with changes in metaphor or technology.

[12] It is important that they do because of the way the Directive is written as a rule (Article 2) with exemptions (Article 3). "This legislator's decision of a 'rule and exemption' approach is of extraordinary importance because the exemptions need to meet very strict application criteria at all times. Thus, the assumption of an exception by way of mere analogies is not convincing" Spranger, T.M. (2015). Legal analysis of the applicability of Directive 2001/18/EC on genome editing technologies. German Federal Agency for Nature Conservation.

[13] Paragraph 8 of Directive 2001/18/EC.

within the coverage of the Directive. The precautionary approach of the Directive, therefore, is the rationale for assuring that both the product is, and only is, modified as intended and that it is safe to release into the environment.

List of conclusions

I have attempted to determine whether the use of oligonucleotide-directed engineering (OGE) is covered by legislation governing the release of genetically modified organisms (GMOs) because its use creates an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.

My conclusion is that OGE creates genetically modified organisms as defined by Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms. This is because all variants of the OGE technique are described by at least Clause 1 or Clause 2 of Annex 1 A part 1 of Directive 2001/18/EC.

I have attempted to determine whether the use of oligonucleotide-directed engineering is exempt from legislation governing the release of genetically modified organisms by Article 3 because it is a form of mutagenesis as described by Annex 1 B of EU Directive 2001/18/EC.

Among other sources, I was able to make use of extensive analyses of similar questions. This includes reports by the Netherlands Commission on Genetic Modification (COGEM) that also summarize the findings of others (COGEM, 2006, 2010). I also drew upon a report prepared by the European Commission New Techniques Working Group (EC, 2012).

My conclusion is that OGE is not included in the list of techniques exempted from Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms.

OGE creates genetically modified organisms because —

- they are at least made using, among other things, recombinant nucleic acid techniques. The Directive was not limited to coverage of a specific named set of techniques and this is indicated by the use of the phrases ‘at least’ and ‘among other things’.
- they have been made using recombinant nucleic acid techniques that form new combinations of genetic material capable of continued propagation in organisms and using oligonucleotides that are recombinant nucleic acid molecules.
- they have been made using oligonucleotides that are nucleic acids that are prepared by whatever means outside of an organism.
- they have been made using nucleic acids, and these are heritable material. All techniques involving oligonucleotides use vectors or methods for direct introduction of the nucleic acid into cells of the distinctive nature of the examples listed in Annex 1 A part 1.

- the technique is based on the use of nucleic acids. These are different from mononucleotides and other kinds of chemical and physical mutagens used in traditional mutagenesis because they are nucleic acids.
- the range of interactions between an oligonucleotide and a genome are substantially different from the range of interactions available to mutagens such as radiation and chemicals, including those that are made from mononucleotides (or nucleosides), because these are not nucleic acids.[14]

Introduction

I was asked to address the question of whether organisms created through the use of oligonucleotide-directed genetic engineering (OGE) are defined by Article 2(2)(a), genetically modified, and/or Article 3, organisms exempted from regulation.

Before proceeding I will define some key terms as they are used in this report.

- Nucleic acid is a linear polymer made from mononucleotides.
- Mononucleotide is a molecule composed of a nitrogenous base, pentose (five carbon) sugar and a phosphate group. The ribose sugar may (RNA) or may not (DNA) have an oxygen atom (more accurately, a hydroxyl group) on the second carbon. A nucleotide is also referred to as a 'base'.
- Nucleoside is a molecule composed of a nitrogenous base and a pentose sugar.
- DNA, deoxyribonucleic acid, is a polymer of (deoxyribo)nucleotides.
- RNA, ribonucleic acid, is a polymer of (ribo)nucleotides.
- Replication refers to the polymerization reactions and the polymerases that make nucleic acids.
- Recombination refers to the reactions and enzymes that promote interactions between nucleic acids, such as oligonucleotides and a chromosome, based on optimization of pairing between nucleotides.
- Protein is a polymer of amino acids.
- *In vitro* (literally 'in glass') means outside of an organism.
- Vector is a delivery (and sometimes also a gene expression) system.

It was beyond my brief to provide a detailed review of OGE or where it has been used in the past. These details, however, are covered to various degrees in the references upon which I base my opinion.

[14] Nucleic acids have 'information' because they are a sequence of nucleotides. Recombination can cause gene conversion, the outcome most common to current uses of OGE. It is defined as the "transfer of DNA sequences between two homologous genes…can be a mechanism for mutation if the transfer of material disrupts the coding sequence of the gene or if the transferred material itself contains one or more mutations" http://ghr.nlm.nih.gov/glossary=geneconversion Access date 27 October 2015.

Directive 2001/18/EC

Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms defines genetically modified organisms in Article 2(2).

2) 'genetically modified organism (GMO)' means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination;

Within the terms of this definition:

(a) genetic modification occurs at least through the use of the techniques listed in Annex I A, part 1;

(b) the techniques listed in Annex I A, part 2, are not considered to result in genetic modification

Annex 1 A part 1

Techniques of genetic modification referred to in Article 2(2)(a) are inter alia:

(1) recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation;

(2) techniques involving the direct introduction into an organism of heritable material prepared outside the organism including micro-injection, macro-injection and micro-encapsulation;

(3) cell fusion (including protoplast fusion) or hybridisation techniques where live cells with new combinations of heritable genetic material are formed through the fusion of two or more cells by means of methods that do not occur naturally.

Annex 1 A part 2

The techniques listed in this section do not create genetically modified organisms.

Techniques referred to in Article 2(2)(b) which are not considered to result in genetic modification, on condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms made by techniques/ methods other than those excluded by Annex I B:

(1) in vitro fertilisation,

(2) natural processes such as: conjugation, transduction, transformation,

(3) polyploidy induction.

Annex 1 B

In order to better refine what does and does not fall within the scope of the Directive, it exempts some techniques or methods in Article 3 by characteristics described in Annex 1 B.

Techniques/methods of genetic modification yielding organisms to be excluded from the Directive, on the condition that they do not involve the use of recombinant

nucleic acid molecules or genetically modified organisms other than those produced by one or more of the techniques/methods listed below are:

(1) mutagenesis,

(2) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods.

The words and phrases used in the Directive have a history (Tooze, 1980-1981) which is important for understanding their meanings. For example, the convention for interpreting Clause 1 of Annex 1 A part 1 was established in 1979. This is discussed in detail below in the section "Capable of continued propagation."

Already by 1980, less than a decade from the world's first recorded *in vitro* DNA recombination experiments, analysts commented on the time and resource commitments behind drafting regulations on recombinant DNA research. "As those many who, in various countries, drafted regulations for this activity discovered, an adequate definition which avoids being scientific nonsense while meeting legal requirements or considerations is not easily found; indeed, if records had been kept of the total time spent by individuals and committees around the world in drafting such definitions, the integral would have to be measured certainly in man-months—perhaps man-years" (Tooze, 1980-1981). This is to say that the Directive's language was not rushed or invented in 2001, but a deliberate and considered choice that has stood debate and challenge (Figure 2).

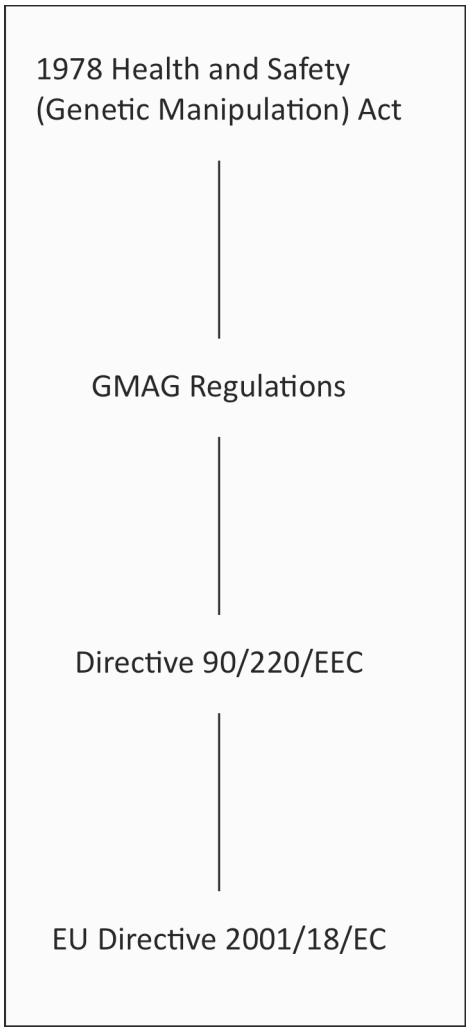

Figure 2. Select significant legislative and regulatory events in the history of the Directive.

Likewise, oligonucleotides were always available for use in recombinant nucleic acid techniques (Itakura and Riggs, 1980). The variety of uses has grown along with the techniques. Indeed, oligonucleotides were being used in recombinant DNA techniques for forms of 'gene editing' and mutagenesis over 20 years before the Directive (Itakura and Riggs, 1980; Rivera-Torres and Kmiec, 2015), belying claims that the newness of such techniques challenges the 2001 Directive (Breyer et al., 2009). Their existence and their use was known to the scientific community and those discussing regulations on genetically modified organisms (Botstein and Shortle, 1985; Shortle et al., 1981). While these techniques were developed during the same period of

time that recombinant DNA safety regulations were also being developed and evolving[15], they did not have a history of safe use in various conventional applications prior to the emergence of regulations.

There is compelling evidence that OGE (and gene editing in general) techniques are older than the Directive. But there is no evidence that the Directive was written to exclude these techniques that had no safety record from conventional use.

The purpose of this report is to attempt to clarify whether the use of oligonucleotides in a method called OGE creates genetically modified organisms and, if so, if it may also be a form of mutagenesis that is exempted from regulation by Annex 1 B of Directive 2001/18/EC.

There is agreement that the 1990 and 2001 Directives were excluding from regulating organisms modified through the use of chemical or radiation mutagenesis (COGEM, 2006; Lusser and Davies, 2013). OGE is substantially different to these forms of mutagenesis. Therefore, OGE is not necessarily covered by the list of exempted techniques provided in Annex 1 B of Directive 2001/18/EC.

In the next section I will discuss pre-existing expert opinions on this and related questions. In the section "Analysis", I will focus on the question of whether OGE is a technique for making genetically modified organisms, and if they would be exempt from regulation as described by Article 3 of Directive 2001/18/EC.

Previous work

Expert bodies such as the EU New Techniques Working Group and the Netherlands Commission on Genetic Modification (COGEM) have previously considered the question of whether genetically modified organisms are produced using oligonucleotide-directed genetic engineering. Those reviews cover uses that fall outside of the scope of this report. However, they also do include uses such as OGE and review other material that is relevant to understanding the context of the questions within the scope of this report. For clarity and completeness, I will present and discuss relevant arguments they have made in Appendix Two.

Analysis

I have considered whether the use of OGE is covered by Article 2(2) of Directive 2001/18/EC and conclude that it is.

I have considered whether the use of OGE is exempt from GMO legislation because it is a form of mutagenesis as described by Annex 1 B of EU Directive 2001/18/EC I have come to the conclusion that OGE is not included in the list of techniques in Annex 1 B.

My conclusion is based on three broad arguments. First, OGE conforms to what Article 2(2) and Annex 1 A part 1 identify as genetically modified organisms and

[15] "the fields of chemical DNA synthesis and recombinant DNA came of age at approximately the same time" Itakura, K., and Riggs, A.D. (1980). Chemical DNA synthesis and recombinant DNA studies. Science *209*, 1401-1405.

procedures that create genetically modified organisms. Second, oligonucleotides are fundamentally unlike traditional chemical and radiation mutagens with a long record of safety prior to the Directive. Third, oligonucleotides are recombinant DNA molecules. As a result, OGE is not a technique that is excluded from coverage of Article 2(2) because it is not described by Article 2(2)(b) nor is it listed in Annex 1 B.

OGE is a procedure that creates genetically modified organisms

The definition of a genetically modified organism is provided by Article 2(2):
'genetically modified organism (GMO)' means an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination

I believe that there is general agreement that this definition applies to OGE (OMM)[16]. However, there is disagreement about whether OGE is covered by GMO legislation. The New Techniques Working Group was split, with a majority arguing that OGE is not captured by Annex 1 A part 1 because oligonucleotides are not recombinant nucleic acids and they are not heritable, and that OGE is a technique of mutagenesis described by Annex 1 B.

A minority of experts reached conclusions similar to those that I have reached. "ODM is a recombinant nucleic acid technique that (i) leads to a new combination of genetic material resulting in a heritable change in the DNA sequence (point 1) and (ii) it involves the direct introduction of heritable material prepared outside of the organism (point 2)" (EC, 2012).

Article 2(2) then goes on to say:
Within the terms of this definition: (a) genetic modification occurs at least through the use of the techniques listed in Annex I A, part 1

Annex 1 A part 1 has three clauses that together provide a general framework for recognizing a genetically modified organism. The words 'at least' reinforce the idea that the list to follow in the Annex is not exclusive or exhaustive, but indicative.

The Directive uses several key phrases in Annex 1 A part 1. My analysis will focus especially on the terms (listed here in the order that they appear in the Annex): *inter alia*; recombinant nucleic acid techniques; produced by whatever means outside of an organism; vector systems; incorporation; propagation; and heritable material.

Inter alia

Recall that part 1 uses the phrase '*inter alia*'. As such, Clauses 1-3 do not constitute an inclusive list that permit other techniques to be excluded by literal reading of terms (Spranger, 2015). For example, combining the 'at least' in Article 2(2)(a), with the '*inter alia*' in part 1, genetic modification occurs through *at least* the techniques listed in Annex 1 A part 1 and *among other things* (Clause

[16] "It seems obvious that OMM [oligonucleotide-mediated mutagenesis] must be considered as leading to genetic modification in the meaning of the EU Directives" Breyer, D., Herman, P., Brandenburger, A., Gheysen, G., Remaut, E., Soumillion, P., Van Doorsselaere, J., Custers, R., Pauwels, K., Sneyers, M., *et al.* (2009). Genetic modification through oligonucleotide-mediated mutagenesis. A GMO regulatory challenge? Environ Biosafety Res *8*, 57-64.

1) recombinant nucleic acid techniques…techniques involving direct introduction of heritable material prepared outside an organism…cell fusion…by means of methods that do not occur naturally.

To argue that a technique does not fall within the description of Clauses 1-3 requires proof that no aspect of the technique is similar to what is described in Clauses 1-3. In contrast, Annex 1 B is an inclusive list that describes exactly what is excluded (Spranger, 2015). Only if all variations of a technique are perfectly described by Annex 1 B will it qualify for exclusion. In other words, the bar is set higher for excluding a technique that causes genetic modification.

Clause 1 terms

Clause 1 describes techniques of genetic modification as including "the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation." I will examine four sub-clauses of the statement:

1. produced by whatever means outside an organism;
2. into any virus, bacterial plasmid or other vector system;
3. incorporation into a host organism; and
4. capable of continued propagation.

Produced by whatever means outside an organism

The first sub-clause indicates that what matters is how the nucleic acid molecule that "produces new combinations of genetic material" was made. If it was made "outside of an organism" (i.e. *in vitro*) then any use of it that can result in new combinations of genetic material makes a genetically modified organism. This would capture all synthesized oligonucleotides of DNA, RNA, a mixture or using non-standard chemistries (Thaler et al., 1996) or nucleic acids derived by *in vitro* enzymatic cleavage from a larger DNA molecule.

Into any virus, bacterial plasmid or other vector system

The second sub-clause describes a non-exhaustive list of means of working with nucleic acids in recombinant nucleic acid techniques, including how to incorporate them into a host and/or genome. Vectors are nucleic acid delivery systems, or 'vehicles' (Chalfant et al., 1979; Luo and Saltzman, 2000). They can be biological (e.g. virus, bacterial plasmid, proteins), chemical and physical (e.g. gold nanoparticle, liposomes, branched and dendritic polymers) vehicles (Chen et al., 2010; Garcia-Chaumont et al., 2000; Gonzalez et al., 1999; Li and Szoka Jr, 2007; Mintzer and Simanek, 2009; Morris et al., 1997; Prevette et al., 2010). If the Directive was exclusive to biological vectors, it did not say so. Moreover, the list is not exclusive to the examples. This clause therefore captures all ways to deliver oligonucleotides into cells.

Some variations of the technique called electroporation may use no biological vector, that is, beyond the oligonucleotide itself.[17] This would still be unusual as

[17] Indeed, the oligonucleotide can be thought of as a vector of the sequences intended to change the recipient.

most procedures use chemicals that conform to the type of chemical vectors above.

In addition, I believe that electroporation can be described as an *in vitro* nucleic acid technique and by extension, a recombinant nucleic acid technique, in part for reasons I set out below in section "Oligonucleotides are recombinant nucleic acids". Electroporation, and chemical delivery systems, also conform to the general description of techniques discussed in Clause 2 (see section "Direct introduction" below). Moreover, electroporation as a technique for creating GMOs using other kinds of recombinant nucleic acid molecules is not disputed.

Incorporation into a host organism

The third sub-clause requires 'incorporation'. It is not clear whether incorporation is used here to mean recombination with, or uptake into, the genome of the host, or just incorporation into the cell of a host organism. The latter interpretation would also be consistent with the processes captured by the definition of a genetically modified organism. The New Techniques Working Group could not come to a consensus on what incorporation meant because some experts agreed "as long as this 'foreign' genetic material is present [in a cell] even if it's not able to replicate the organism should be considered a GMO" (EC, 2012). This is consistent with incorporation being entry into the cell.

There is nothing in the Directive to indicate that a nucleic acid introduced into a cell had to become inserted into a chromosome in the genome to make the organism genetically modified. Certainly the uptake of plasmid or virus into a bacterium is considered to be a process that makes a genetically modified organism and incorporation into an existing genomic molecule is not necessary in many cases for inheritance. *I conclude that uptake into a cell is sufficient to qualify as 'incorporation'.*

Capable of continued propagation

The fourth sub-clause is ambiguous because it is preceded by an ambiguous pronoun. In context, the clause reads: "but in which *they* are capable of continued propagation" (emphasis added). The pronoun 'they' might refer to the "nucleic acid molecules produced by whatever means." However, this would confer the capability of propagation onto the nucleic acid itself, as in "but in which [nucleic acids produced by whatever means] are capable of continued propagation". That meaning would be inconsistent with how the Directive defines organisms. The Directive defines organisms in Article 2(1) as "any biological entity capable of replication or of transferring genetic material". The Directive does not define propagation as a property of nucleic acids but instead as a property of organisms.

Hence, 'they' most likely instead refers to organisms with "new combinations of genetic material". Because it is new combinations of genetic material that are critical features of genetically modified organisms — regardless of whether the new combinations arise from insertions into, or deletions of existing parts of the genome[18] — I believe that the Directive should be interpreted this way: "the

[18] This was an outcome of recombinant nucleic acid techniques established well before the Directive was written and at about the same time as the GMAG guidelines were written. See:

formation of new combinations of genetic material…in which they do not naturally occur but in which they are capable of continued propagation."

For example, where an oligonucleotide was used to create a deletion of material from a genome, the end result would still be genetic material that had been altered through a process that did not occur naturally by mating or natural recombination, and thus the resulting organism would be genetically modified.

One other piece of evidence gives me confidence that 'they' refers to organisms with "new combinations of genetic material". The clause —

Techniques of genetic modification referred to in Article 2(2)(a) are inter alia:

(1) recombinant nucleic acid techniques involving the formation of new combinations of genetic material by the insertion of nucleic acid molecules produced by whatever means outside an organism, into any virus, bacterial plasmid or other vector system and their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation

derives nearly word for word from the United Kingdom's recombinant DNA guidelines written by the Genetic Manipulation Advisory Group (GMAG) in the late 1970s.[19] It sparked controversy even then. *Nature* magazine reported in March 1979—

The chief difficulty, says Dr Sherratt, arises in the meaning assigned to 'they' in the phrase 'in which they do not naturally occur'. If it refers to the 'new combinations', then all self-cloning experiments are included in the guidelines. If, on the other hand, it refers to the 'nucleic acid molecules', as most scientists have assumed it does, then all self-cloning experiments are exempt from regulation. This is the point that GMAG has recently been debating (Redfearn, 1979).

By April of that same year, GMAG confirmed that self-cloning experiments, where fragments of DNA from the same organism were rejoined using *in vitro* recombinant DNA techniques, were to be regulated (Anonymous, 1979). This established the regulatory convention that the 'they' referred to 'new combinations' in the organism, not the nucleic acid molecules used in the procedure. (GMAG did make a distinction at the risk assessment level, however, allowing some self-cloning experiments to be subject to a less stringent risk assessment and risk management framework.[20])

Scherer, S., and Davis, R.W. (1979). Replacement of chromosome segments with altered DNA sequences constructed in vitro. Proc Natl Acad Sci USA *76*, 4951-4955.

[19] For example, the phrasing of Annex 1 A part 1(1) appears to be homologous to: "Genetic manipulation" is defined as "the formation of new combinations of heritable material by the insertion of nucleic acid molecules, produced by whatever means outside the cell, into any virus, bacterial plasmid, or other vector system so as to allow their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation." Health and Safety Regulations (Genetic Manipulation), 1978, Reg. 2 Fancher, M.V. (1985-1986). Deliberate environmental release of genetically modified organisms: a comparative analysis of British and United States regulations. *9*, 77-106. The word changes are "Genetic manipulation" to "Techniques of genetic modification", "the cell" to "an organism" and "heritable material" to "genetic material".

[20] GMAG decided that some self-cloning experiments no longer needed to be notified. "In general, however, self-cloning experiments are still considered to be within GMAG's remit. 'The group is satisfied' says the statement 'that many self-cloning experiments are without conceivable

Meanwhile, the equivalent guidelines being produced contemporaneously in the United States explicitly differed with GMAG on this point (Walgate, 1979). The debate by scientists in the two different countries over this difference in guidelines reveals that the GMAG decision was to deliberately include the use of rejoined DNA fragments from the same organism as qualifying as a 'recombinant nucleic acid technique'.

GMAG made a conscious decision to capture rejoined molecules from the same organism in the definition of organisms to be regulated as genetically modified. Over 20 years later, the Directive purposefully preserved the same words in Annex 1 A part 1. This history establishes an unbroken chain of regulatory convention which emerged in the UK before 1980 and transferred to the European Union in 2001. Recombinant DNA molecules do **not** have to be combinations of "sequences that do not naturally occur next to each other" (COGEM, 2010).

Placing the emphasis on the new combinations of genetic material being in the genetically modified organism and not in the recombinant nucleic acid molecules is also consistent with the Cartagena Protocol on Biosafety[21] (IUCN, 2003). Article 3(g) of the Protocol defines a living genetically modified organism (LMO) as: "'Living modified organism' means any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology" (CBD, 2003), rather than an organism that possesses an introduced molecule that is a novel combination of genetic material.

Directive 2001/18/EC gives the Protocol status, saying that the "content of this Directive duly takes into account international experience in this field and international trade commitments and *should respect the requirements of the Cartagena Protocol on Biosafety to the Convention on Biological Diversity*" (emphasis added).

The nucleic acid used in OGE can be but does not have to be the source of the novel combination of heritable material in organisms that are in the coverage of the Directive. Rather it is sufficient for the nucleic acid to be part of the process through which a novel combination of heritable material is created.[22] Using this interpretation, much of the unease that comes with assigning an arbitrary quantitative degree of dis-similarity of the oligonucleotide sequence from the host is avoided (Appendix Two).[23]

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hazard', but it 'interprets the definition of genetic manipulation to cover self-cloning experiments'" Anonymous (1979). GMAG states the position on 'self-closing'. Nature *278*, 496.

[21] https://bch.cbd.int/protocol. Access date 8 October 2015.

[22] COGEM presents a contrary view, as in, for example: "Within this context, the sequence of the oligonucleotide is the central issue: does the oligonucleotide contain a combination of sequences that do not naturally coexist?" COGEM (2010). The status of oligonucleotides within the context of site-directed mutagenesis. COGEM advice and report. CGM/100701-03.; "Considering the above, the following points are important when answering the question when an oligonucleotide should be seen as a recombinant nucleic acid…Furthermore, a point mutation in a sequence cannot be considered a recombination of different sequences" ibid.

[23] As an aside, COGEM also argues that the Protocol emphasis on novel combinations being in the GMO rather than the recombinant nucleic acid is a bridge to 'product based' rather than 'process based' legislation. "By increasing the emphasis on the fact that the plants or crops need to have new properties, a connection is made with 'product based' legislation" ibid. I disagree at least in

Clause 2 terms

If it is possible to conceive of a highly specialized procedure where the conditions of Clause 1 would not apply to the use of oligonucleotides or common variations of the OGE procedure, then these in any case would be captured by Clause 2. This clause of Annex 1 A part 1 describes techniques of genetic modification as including those "techniques involving the direct introduction into an organism of heritable material prepared outside the organism including [but not limited to] micro-injection, macro-injection and micro-encapsulation."

Heritable material

On the question of what is meant by 'heritable material' as it appears in Clause 2 of the Directive, the European Commission New Techniques Working Group was agreed that 'heritable' was the equivalent of 'genetic', but divided on whether heritable material had to be inherited to qualify as being heritable.

Although heritable material is not clearly defined in the Directive, there are two possible interpretations: i. "heritable material" must be inherited in the case in question. The argument being that the first indent in the list of Annex IA Part 1 involves the use of vectors and refers to the transfer of genetic material into a host organism and continued propagation. In order for this to be consistent with the second indent in the list of Annex IA Part 1 heritable material should be interpreted as being propagated through the host organism and not just being transiently present (see section 4.4); and, ii. "heritable material" has simply to be capable of being inherited (EC, 2012).

The majority indicated that heritable material was a term that required inheritance, but they did not say why. Within the normal parlance of science, both interpretations are common. Heritable material is used as a synonym of genetic material and it is not common to find scientists referring to DNA as a gene only after it has replicated; they refer to DNA as genetic material because genes are made of it.

I have found no evidence to suggest that the Directive places the additional technical restriction on the term that the majority in the working group preferred. Indeed, to assume that it does would be equal to assuming that the Directive was distinguishing between many different kinds of DNA molecules, such as those with replication origins but without sequences that ensured stable and equal segregation during cell division, those with both but that only work in some species or any number of other specific attributes that might nuance observations of inheritance.

Whether genetic material is heritable depends on many attributes including the receiving organism. Not all of these can be evaluated *a priori* but could be in a risk assessment on a case-by-case basis.

For example, DNA molecules without known replication-directing sequences in an organism can nevertheless at least sometimes achieve some level of replication and thus the potential to be passed on, even if intermittently, to descendants (Srivastava and Ow, 2003).

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the sense that COGEM uses 'product', because the Protocol does not require the outcome of the modification to be a change in properties of the organism, only a change in properties of the genome IUCN (2003). An Explanatory Guide to the Cartagena Protocol on Biosafety.

An experiment in 1990 showed that the cloning plasmid pBR322, which is only known to replicate in some bacteria, could by *any* of 10 unlinked small sequence changes be converted into a plasmid that replicated very well in eukaryotic cells (Kipling and Kearsey, 1990). The authors concluded "that changes in replication origin distribution may arise de novo by point mutation" (Kipling and Kearsey, 1990). The level of successful replication is a matter of technological capacity to detect the products of replication. That will vary by organism and cannot be assumed knowledge for all organisms.

Another example is common in bacterial genetics. Bacteria are 'transiently' transformed using DNA that encodes a transposase that may act on a transposon, cause its mobilization and re-insertion without retention of the gene for the transposase, resulting in formation of new combinations of genetic material (used by Cooper and Heinemann, 2000; Heinemann et al., 1996). The disappearance of the DNA corresponding to the transposase gene did not make the effects of the transposon not inherited. It is as unlikely that the Directive was written to exclude such possibilities from consideration.

Moreover, some molecules become inheritable by insertion mediated by recombination with another molecule of the genome. The intention of many applications of OGE are recombination events leading to gene conversion (see footnote 14 for a definition). What matters in the case of genes is the order of nucleotides, not the particular atoms of a molecule of DNA at some point in time. There is almost no 'material' transfer across generations of organisms, but instead information transfer. We do not have the atoms of the DNA in our ancestors, but we do still have with few exceptions the same sequence of nucleotides in our DNA molecules. Regardless of whether recombination events result in 'insertion' of the oligonucleotide molecule or insertion of the sequence information transferred from the oligonucleotide molecule, the end result is a new combination of genetic material.

DNA is often referred to as heritable material, and DNA is a nucleic acid. 'Heritable' is clearly an adjective[24] that describes the known, incontrovertible *potential* of nucleic acids to participate in reactions that result in replication, which in turn creates the potential for their passage to subsequent generations[25]. This potential may be realized if the molecule interacts with the enzymes necessary for DNA replication, or if it recombines with any other nucleic acid that can. The ability to recombine is inherent in it being a nucleic acid. These are nontrivial distinctions from other kinds of molecules that do not have this potential. The distinctions are biologically relevant.

[24] To be read in the same was as 'flammable material', which does not have to be on fire to be regulated.

[25] From the Merriam-Webster dictionary: "capable of being inherited or of passing by inheritance" http://www.merriam-webster.com/dictionary/heritable. Access date 29 October 2015. This point and relevant jurisdiction interpretations is also covered by Dr T.M. Spranger in his legal opinion: "This is not only illustrated by the German official translation of Directive 2001/18/EC which merely refers to "Einführung von Erbgut in einen Organismus" but by the English version of the Directive: the sole qualification of the material as inheritable does not imply that this potential must come to light at a certain point in time or in a certain manifestation" Spranger, T.M. (2015). Legal analysis of the applicability of Directive 2001/18/EC on genome editing technologies. German Federal Agency for Nature Conservation.

Further evidence that 'heritable material' is often used as a synonym of 'genetic material' comes from the descent by modification of the language used in the Directive. As described above (see footnote 19), the Directive language derives from the United Kingdom's 1978 Health and Safety (Genetic Manipulation) Act where the term 'heritable material' has changed to 'genetic material' in the Directive.

The meaning of "direct introduction into an organism of heritable material prepared outside the organism" unambiguously applies to oligonucleotides because such genetic material is hereditary material.[26] Genetic material is also commonly used as a synonym of genetic *information*.[27] The sequence of nucleotides is information. The sequence information in an oligonucleotide used in OGE is inherited by transfer of the sequence information from one molecule to the other, even if sometimes there is no physical transfer of material (Thaler et al., 1996). If the introduced mutations are passed down, OGE induced heritable change through "new combinations of genetic" information.

The use of oligonucleotides involves the direct introduction into an organism of heritable material prepared outside the organism and thus is consistent with a characteristic of techniques that produce organisms that should be regulated as genetically modified.

Direct introduction

Clause 2 is not an exhaustive list of direct introduction techniques because the list is preceded by the qualifiers "at least" and "*inter alia*". As introduced briefly above, procedures involving electroporation and chemical delivery cause direct introduction of heritable material, oligonucleotides, into a cell either by creating membrane-permeating channels through which DNA can flow through, or by encapsulating the nucleic acid.

Direct introduction techniques are not limited to the few listed in Clause 2.

Electroporation creates a physical puncture in the membrane using electricity instead of the metal of needle in an injection or the

[26] For example, this definition from the United States National Institutes of Health. "DNA, or deoxyribonucleic acid, is the hereditary material in humans and almost all other organisms" http://ghr.nlm.nih.gov/handbook/basics/dna. Access date 29 October 2015. Theodosius Dobzhansky wrote in Encyclopedia Britannica: "genes, the functional units of heritable material" http://www.britannica.com/science/heredity-genetics. Access date 29 October 2015. The Merriam-Webster Dictionary links heritable with hereditary: "passed or able to be passed from parent to child before birth" and "genetically transmitted or transmittable from parent to offspring" http://www.merriam-webster.com/dictionary/hereditary.
Moreover, the New Techniques Working Group also acknowledged the legitimacy of this definition of heritable material in context of the Directive EC (2012). New Techniques Working Group *Final Report*. European Commission.

[27] "The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T)…The order, or sequence, of these bases determines the information available for building and maintaining an organism, similar to the way in which letters of the alphabet appear in a certain order to form words and sentences" http://ghr.nlm.nih.gov/handbook/basics/dna. Access date 29 October 2015. Also "DNA and RNA are the polynucleotides known to carry genetic information in life" Thaler, D.S., Liu, S., and Tombline, G. (1996). Extending the chemistry that supports genetic information transfer in vivo: phosphorothioate DNA, phosphorothioate RNA, 2'-O-methyl RNA, and methylphosphonate DNA. Proc Natl Acad Sci USA *93*, 1352-1356.

carrier particles fired into cells during the biolistics procedure. Indeed, in scientific discussions, electroporation may be discussed along with injection as alternative techniques for DNA delivery (Davey et al., 1989; Liu et al., 2014).[28]

Oligonucleotide encapsulation techniques include chemical treatments, such as the use of liposomes (Bailey and Sullivan, 2000) or polymers such as poly(lactide-co-glycolides) (Freytag et al., 2000).

There may be variations on techniques that make them more or less literally similar to the language in Clauses 1-3 of Annex 1 A part 1. If a member of the family of variants of a technique fits the general description, the technique is included. Any other way of managing the Directive would not be practical.

Conclusion: if oligonucleotides are produced by whatever means outside of an organism and incorporated into a host organism, the host becomes a genetically modified organism. It is not the retention of the oligonucleotide in the genome of the host that determines if the product is a genetically modified organism, but whether the resulting change in combinations of genetic material in the host continue to propagate. The point is that a nucleic acid was produced by whatever means outside of a cell and is then used to cause a change in an organism. Any nucleic acid produced by whatever means outside of an organism is a recombinant nucleic acid and has been regulated as such since the 1970s. The resulting change in the sequence of nucleotides in genomes brought about by the use of a recombinant nucleic acid molecule is what matters for classifying an organism as a regulated genetically modified organism.

Even if a rare variant of the OGE procedure could be imagined to not conform to the description in Annex 1 A part 1(1), oligonucleotides are nucleic acids which are heritable material and would therefore be captured by Annex 1 A part 1(2). Moreover, the techniques described in Annex 1 A part 1(2) for direct introduction also describe the techniques used for direct introduction of oligonucleotides.

Oligonucleotides are unlike traditional chemical and radiation mutagens

I have two broad arguments. One, oligonucleotides are unlike chemical and radiation mutagens because they do not have a long record of safety arising from conventional use.[29] Second, they are fundamentally different chemically and biologically from chemical mutagens that have conventionally been used.

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[28] "The first *in vitro* and *in vivo* attempts to utilize electrotransfer for gene delivery were demonstrated in 1982 and 1991, respectively. Compared with plasmid DNA (pDNA) injection alone, addition of electric pulse drastically increases transgene expression and reduces the inter-individual heterogeneity on gene expression" Liu, S., Ma, L., Tan, R., Lu, Q., Geng, Y., Wang, G., and Gu, Z. (2014). Safe and efficient local gene delivery into skeletal muscle via a combination of pluronic L64 and modified eletrotransfer. Gene Ther *21*, 558-565. "Thus, many have tried to circumvent endocytotic uptake mechanisms through mechanical means such as electroporation or microinjection or through agents thought to aid in membrane permeation" Prevette, L.E., Mullen, D.G., and Banaszak Holl, M.M. (2010). Polycation-induced cell membrane permeability does not enhnace cellular uptake or expression efficiency of delivered DNA. Mol Pharm *7*, 870-883.

[29] This is not to be confused with an unblemished record of safety. For example, a modern automobile can be determined to be safe if used properly but that does not mean every car model

Safety record

Whereas this Directive should not apply to organisms obtained through certain techniques of genetic modification which have conventionally been used in a number of applications and have a long safety record.[30]

Directive 2001/18/EC is the immediate descendant of Directive 90/220/EEC (23 April 1990). Directive 90/220/EEC made a distinction between genetically modified organisms with a 'long safety record' and those that are produced at least by (but not only these) and among other things the recombinant nucleic acid techniques described in Clause 1, or using direct introduction methods such as those described in Clause 2, of Annex 1 A part 1. That distinction was maintained without modification in Directive 2001/18/EC.

OGE did not have a long safety record derived from conventional use at the time the Directive was written.

In addition to common language in the chapeau[30], genetically modified organisms explicitly exempted were described in Annex 1 B of both Directives, with one significant difference in description (Table 1). The only substantive difference between the phrasing of 1990 and 2001 is the addition of 'recombinant nucleic acid molecules' as a further disqualification from the exemption provided by Article 3 of the Directive.

Oligonucleotides were available as research reagents already in the 1970s. Coincident with the emergence of the techniques of genetic manipulation was the capacity to chemically synthesize oligonucleotides of desired sequence (Shortle et al., 1981). Hence, oligonucleotides either synthetic or isolated using other kinds of *in vitro* nucleic acid methods had been in existence during the development of all iterations of recombinant DNA safety regulations and were among the tools of the techniques being discussed contemporaneously with the development of regulations (Botstein and Shortle, 1985; Shortle et al., 1981).

Their use, including to introduce new combinations of genetic material (including *in vivo*), was well established in the laboratory, but not in food, agriculture or medicine.[31] Even by 2001 there were, to my knowledge, no commercial products made by OGE and therefore upon which to build a long safety record from conventional use. It would accordingly be no surprise to find that their use in recombinant nucleic acid techniques is captured by one or both of the Directives.

Recital paragraph 2 of Directive 2001/18/EC says that at least in part the purpose of updating from Directive 90/220/EEC was that: "There is a need for

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was safe, as recalls show. Instead, I think this means that at the time of the Directive, there was reason to think that products arising through these techniques were generally safe or expected to made so through other means outside of the scope of the Directive.

[30] Chapeau of Directive 90/220/EEC. Recital paragraph 17 of Directive 2001/18/EC reads similarly as: "This Directive should not apply to organisms obtained through certain techniques of genetic modification which have conventionally been used in a number of applications and have a long safety record."

[31] OGE still does not have such a record. I am aware of only one genetically modified organisms produced using OGE that has so far been approved for release into the environment, and none that have been so far released at commercial scale. If there are some and they been released following a risk assessment of the type described by Annex II of the Directive, they will not have been in commercial use for long.

clarification of the scope of Directive 90/220/EEC and of the definitions therein." A difference between the year 1990 and the year 2001 was that Annex 1 B was upgraded in 2001 with the term 'recombinant nucleic acid molecule'. The upgrade does not cause previously exempted organisms to become regulated. It seems unlikely that this upgrade was needed to remove ambiguity over 'transgenes' because these were adequately captured already by the 1990 EU Directive and regulations in member states preceding that Directive. Furthermore, I have not found any fundamentally new recombinant nucleic acid procedures introduced between 1990 and 2001.

Table 1. Annex 1 B

Directive 90/220/EEC	Directive 2001/18/EC
Techniques of genetic modification to be excluded from this Directive, on condition that they do not involve the use of GMOs as recipient or parental organisms, are: *(1) mutagenesis,* *(2) cell fusion (including protoplast fusion) of plant cells where the resulting organisms can also be produced by traditional breeding methods.*	*Techniques/methods of genetic modification yielding organisms to be excluded from the Directive, on the condition that they do not involve the use of recombinant nucleic acid molecules or genetically modified organisms other than those produced by one or more of the techniques/methods listed below are:* *(1) mutagenesis,* *(2) cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods.*

The inclusion of the new phrase into Annex 1 B therefore served to ensure that as techniques changed (or their descriptions changed) they did not become unintentionally exempted. The phrase 'recombinant nucleic acid molecule' provides surety that oligonucleotides and other natural or synthetic nucleic acids, that were prepared by whatever means outside of a cell and which could be used in nucleic acid techniques including those that resulted in creating mutations, were not excluded from coverage of Directive 2001/18/EC.

This interpretation is consistent as well with the updated National Institutes of Health (NIH) Guidelines definitions.

In the amended NIH Guidelines, recombinant and synthetic nucleic acid molecules are defined as:

(i) molecules that a) are constructed by joining nucleic acid molecules, and b) can replicate in a living cell (i.e. recombinant nucleic acids);

(ii) nucleic acid molecules that are chemically or by other means synthesized or amplified, including those that are chemically or otherwise modified but can base pair [hybridize] with naturally occurring nucleic acid molecules (i.e. synthetic nucleic acids); or

(iii) molecules that result from the replication of those described in (i) or (ii) above. [32]

NIH defines oligonucleotides as recombinant or synthetic molecules. While NIH captures these molecules in its definitions, it exempts them from further risk assessment in many kinds of experiments.[33]

The discussion in the section "Oligonucleotides are recombinant nucleic acids", reinforces the conclusion that OGE was not a form of engineering that was meant to be exempted from regulation.

Spectrum of changes

OGE cannot be both a new technique unanticipated by the Directive and have products that "have conventionally been used in a number of applications and have a long safety record". An examination of the spectrum of genetic changes that can be brought about by OGE reveals that it is also different from the spectrum of changes created by mutagenesis techniques that pre-date the use of recombinant nucleic acids.

OGE cannot be both a new technique and a technique with a long safety record.

The application in one form or another of oligonucleotides *in vivo* resulting in hybridization to a target DNA sequence pre-dates the Directive, but the set of organisms that have been subject to either a formal scientific risk assessment or benefitted from a history of use that might provide a long safety record in the environment is empty. However, some are tempted to see this contradiction reconciled by claiming that the techniques are new (and thus not in the scope of Directive 2001/18/EC) but so much like other techniques that were excluded (for whatever reason, see footnotes 8 and 29) that they should be considered like some techniques listed in Annex 1 B.

This reasoning fails on two counts. First, that two processes can produce similar products does not mean that all products of the two processes are the same and only the same (Figure 1). Second, it ignores differences between what may be interesting scientific questions from what are relevant social issues, recalling that Directive 2001/18/EC is not regulation based solely on scientific matters but also ethical and socioeconomic.[34]

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[32] http://osp.od.nih.gov/sites/default/files/NIH_Guidelines_0.pdf. Access date 7 October 2015. "Those synthetic nucleic acids that: (1) can neither replicate nor generate nucleic acids that can replicate in any living cell (e.g., oligonucleotides or other synthetic nucleic acids that do not contain an origin of replication or contain elements known to interact with either DNA or RNA polymerase), and (2) are not designed to integrate into DNA".

[33] Oligonucleotides that are not designed to integrate into replicating molecules or replicate independently are exempt from the NIH Guidelines. As OGE does result in integration of all or part of the oligonucleotide, this procedure may be regulated in some circumstances. Moreover, the NIH Guidelines say that other restrictions may also apply depending of federal agency or State.

[34] Council Conclusions of December 2008 (16882/08): cf. (ii) Appraisal of socio-economic benefits and risks "POINTS OUT that under Directive 2001/18/EC, the Commission is to submit a specific report on the implementation of the Directive, including an assessment, inter alia, of socio-economic implications of deliberate releases and placing on the market of GMO."

Genetic spectrum

The scientific details of how OGE works become relevant to the first issue of physical description of the products. The range of nucleotide changes caused by OGE and 'conventional' mutagenesis can be different. For example, consider an attempt to change a DNA sequence from G**G**AT…C**A** to G**A**AT…C**C**. The use of the classical mutagen ethyl methylsufonate (EMS) results in G:C to A:T transitions. Use of this mutagen could cause one but not both changes. Another kind of mutagen, perhaps 2-amino-6-methylaminopurine, would be needed to create the second A to C transversion mutation. The probability of these two closely linked changes by a non-targeted mutagen is low, and the rarity of a genome with that particular change would the same as any genome with any particular combination of target change and unintended change. The probability of a product with the **A**AT**…**C**C** sequence and any other particular unintended change is much lower still.

Due to the more targeted nature of the oligonucleotide, the efficiency of change at unintended sites may make such changes more probable than using other mutagens that make more location random changes. In the screening process that follows mutagenesis (Segal and Meckler, 2013), genomes with unintended changes are rare because the probability of two changes is the product of the probability of the particular change and probability of a second change in the same genome.

In contrast, the frequency of the intended two nucleotide change by OGE is very high.[35] Thus, it is more likely that any product with this change will have a second unintended ('off-target') change. The oligonucleotide will effect the change at any other unintended site to which the oligonucleotide might bind and which also has a different sequence (Nyergers et al., 2014).

Several oligonucleotides can be delivered at once to a target cell to cause changes at different locations or assemble *in vivo*, or OGE can be applied serially to a single organism or its descendants to achieve a fundamental re-writing of significant portions of its genome (Carr and Church, 2009).[36] The outcomes are thus no less distinctive than what can be obtained using a transgene. This is not achievable by techniques such as chemical or radiation mutagenesis because they lack the qualities that make OGE 'new' in a scientific sense.

Hybridization is an ability special to oligonucleotides that makes them able to also participate in sorts of reactions that do not occur when other kinds of mutagens are used.[37] An obvious case in point is the activity of the endogenous

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[35] Using a single or possibly multiple oligonucleotides to create the respective changes Isaacs, F.J., Carr, P.A., Wang, H.H., Lajoie, M.J., Sterling, B., Kraal, L., Tolonen, A.C., Gianoulis, T.A., Goodman, D.B., Reppas, N.B*., et al.* (2011). Precise Manipulation of Chromosomes in Vivo Enables Genome-Wide Codon Replacement. Science *333*, 348-353.

[36] "In addition, when assessing new techniques it has to be taken into account that the respective procedures can be applied one after another with the effect that in the end, extensive modifications up to the substitution of the whole genome of the target organism can be reached" Spranger, T.M. (2015). Legal analysis of the applicability of Directive 2001/18/EC on genome editing technologies. German Federal Agency for Nature Conservation..

[37] The definition of hybridization from the United States National Institutes of Health makes clear that polymers of nucleotides and not individual nucleotides possess the property of hybridizing. "Hybridization is the process of combining two complementary single-stranded DNA or RNA

nucleases that create double-stranded breaks using an oligonucleotide as a guide.

Whether or not OGE produces any particular product that is more liable to cause harm in the environment is not the characteristic that determines whether the product is subject to regulation under Directive 2001/18/EC. Genetically modified plants can pass risk assessments of the type required by the Directive as shown by the approval of the genetically modified maize MON810 and other genetically modified products. The only question of relevance is whether an unsafe product made through a process such as OGE could be inadvertently released because it was exempted from the mandatory risk assessment of regulated organisms.

Socioeconomic spectrum

Attempting to extrapolate the characteristics of products of conventional mutagens to the untested products of OGE breaks down further when ethical and socioeconomic issues are considered. Consider, by way of example, the Cibus Company patent on the Rapid Trait Development System (RTDS) (Gocal, 2014). Here it is argued that the products of the RTDS are indistinguishable from the products of mutagenesis techniques that are not regulated[38], but still different enough to qualify for an instrument of intellectual property protection based on novelty.

In the sphere of socioeconomics, protection of a product by a utility patent is significantly different from protection by other weaker forms of intellectual property. These differences manifest both in relationships between competing innovators[39] and in relationships between suppliers and consumers. The application of utility patents to agricultural germplasm neither has a long safety record nor is it without evidence of adverse effects (Heinemann, 2007; IAASTD, 2009).

Moreover, to my knowledge all commercially released genetically modified plants have or have had utility patents associated with them. This is different from the products other breeding techniques listed in Annex 1 B which rarely receive such powerful intellectual property protections. In joining the set of products that invariably receive such protection, OGE by RTDS is more like classical genetic engineering than like conventional mutagenesis.

Oligonucleotides are chemically and biologically unlike traditional chemical mutagens

ODM stands for oligonucleotide-directed mutagenesis. By reference to the term alone, a homology is implied between OGE and methods used to create mutations in organisms that are exempted from the GMO legislation by Annex 1 B (1). As discussed in the previous section, OGE was not a procedure with a long history of use as were chemical and radiation mutagens at the time the Directive

molecules and allowing them to form a single double-stranded molecule through base pairing" http://ghr.nlm.nih.gov/glossary=dnahybridization.

[38] "These technological breakthroughs produce precise and predictable results with beneficial traits that are indistinguishable from those developed through traditional plant breeding, but with faster results" http://www.cibus.com/technology.php Access date 24 October 2015.

[39] http://www.cibus.com/press_release.php?date=042512 Access date 24 October 2015.

was written, especially not for applications involving plants (COGEM, 2006; Lusser and Davies, 2013). The similarity in terms may help scientists communicating with each other about certain experimental details, but it is not taken for granted that the techniques are related by variation from a common fundamental property (Spranger, 2015).

It may be the intention of a scientist to cause a genetic modification, but that does not make the process a technique of mutagenesis referred to in Annex 1 B(1). Intentions of the scientist neither endows molecules with properties they do not otherwise have nor restricts their activities to only those that are desired by the scientist. For example, regardless of why an oligonucleotide might be used, it has the inherent property of being able to hybridize with DNA. This ability is unique to polymers of nucleic acids or analogues of nucleic acids (Appendix Two).

COGEM's decision to use the length of a molecule, as in number of bases, as a defining characteristic brought it to the conclusion that a precise definition of oligonucleotides was neither relevant nor desirable (Appendix Two). COGEM recommended "that for GMO legislation, the maximum length of an oligonucleotide should be defined as approximately 120 nucleotides" (COGEM, 2010). The use of larger nucleic acid molecules would make the product a regulated genetically modified organism.

However, what I believe has stymied COGEM's attempt to achieve a satisfactory definition is fixation with length and ever evolving limits of technology. Taking a step back from COGEM's framework affords a different perspective that would also provide regulatory consistency. The one theme that appears to me to be relevant is that by definition *oligonucleotides are molecules composed of more than one nucleotide*.

All oligonucleotides are nucleic acids. That endows them with properties special to nucleic acids.

This may at first appear to be as arbitrary as COGEM's definition of oligonucleotides, where it attempts to distinguish between molecules of up to 120 nucleotides and those 121 nucleotides and longer. However, it is not. *Whereas a large variety of molecules have been called either oligonucleotides or recombinant nucleic acids by scientists, never is a mononucleotide (or single nucleoside) called either an oligonucleotide or a recombinant nucleic acid molecule.* The critical difference is that a nucleotide (or nucleoside) is not a nucleic acid[40]; only a covalent linkage between nucleotides results in the formation of a nucleic acid.

A mononucleotide (or a nucleoside, base) may be a mutagen and they had a long history of use at the time that the Directive was written. Mononucleotides used as mutagens[41] are incorporated through reactions that create phosphodiester linkages between nucleotides, the act of polymerization. The primary

[40] For example, this definition from the United States National Institutes of Health makes clear that the nucleic acids DNA and RNA are polymers. "DNA and RNA have great chemical similarities. In their *primary structures* both are linear polymers (multiple chemical units) composed of monomers (single chemical units), called nucleotides…**Polymerization of Nucleotides Forms Nucleic Acids**". http://www.ncbi.nlm.nih.gov/books/NBK21514/. Access date 8 October 2015.

[41] For example, 5-bromouracil, inosine, 2-aminopurine or 2-amino-6-methylaminopurine.

distinguishing characteristic of the 'new' oligonucleotide-directed genetic engineering techniques is the ability to hybridize polymers of nucleic acids *in vivo*, and thus stimulate reactions that result in the transfer of sequence order information from one polymer (or strand) to another.[42]

The length of the oligonucleotide may influence specificity and efficiency, but not change the fundamental property that makes OGE both 'new' and commercially desirable. In a study comparing the specificity and efficiency of an oligonucleotide and a plasmid to effect site-specific change of a target nucleotide in a chromosome, both donor DNAs performed equally well (Wagner et al., 2010). The plasmid was ~5,000 base-pairs long and clearly a recombinant nucleic acid. It was introduced into the cell using the same *in vitro* nucleic acid techniques as the 41 base-pair oligonucleotide and caused the same changes *in vivo*.

Lastly, as recombinant nucleic acid techniques including the *in vitro* use of oligonucleotides to change DNA sequences were being developed, they were seen by the scientists of the day to be different from classical (traditional) mutagenesis. As an authoritative review in the prominent journal *Science* said—

The considerable increases in mutagenic efficiency and specificity attainable with the new methods [which included techniques called ODM], however, do exact a price. Because these methods are designed for use on isolated DNA molecules, a gene must almost always be removed from its normal genetic context- a unique locus on a large complex chromosome inside a living cell (or virus)- and inserted into the abnormal context of a small cloning vector propagated in E. coli*. Unlike classical* in vivo *mutagenesis, in which all mutations are isolated* in situ, in vitro *mutagenesis invariably yields gene mutations out of their normal context. This is the most radical and most troublesome difference between the classical methods and the powerful* in vitro *methods (Botstein and Shortle, 1985).*

Conclusion: The history of the Directive provides evidence that the term 'mutagenesis' in Annex 1 B referred to organisms modified by techniques that had a long safety record already by at least 1990. Procedures using oligonucleotides to cause sequence changes were in existence by then, and thus regulators of the day would have been aware of them, but techniques using oligonucleotides did not have a long safety record from conventional use.

Furthermore, I have found a way to make a credible and reasonable distinction between mutagenesis techniques that use mononucleotides and mutagenesis techniques that use oligonucleotides. This distinction was relevant at the time the Directive was written, because the use of mononucleotides as mutagens was well known but the use of oligonucleotides was relatively new. There is no larger difference between how mononucleotides are used biologically (or chemically) than what is encountered at the division between nucleotides and nucleic acids. This is

[42] Oligonucleotide-mediated gene modification, a synonym of OGE, "is believed to encompass two major steps, strand pairing and gene conversion" Laible, G., Wagner, S., and Alderson, J. (2006). Oligonucleotide-mediated gene modification and its promise for animal agriculture. Gene *366*, 17-26.

illustrated by the unique ability to hybridize oligonucleotides to other nucleic acids (both *in vivo* and *in vitro)*.

Oligonucleotides are recombinant nucleic acids

If oligonucleotides are 'recombinant nucleic acid molecules' then OGE does not qualify for exemption under Article 3 because of the wording in Annex 1 B. Scientists historically have considered oligonucleotides to be a form of recombinant DNA (a kind of nucleic acid). From the work I have undertaken, I conclude that regardless of nucleotide sequence oligonucleotides are 'recombinant nucleic acid molecules'.

What is recombinant? COGEM concluded that recombinant nucleic acids are composed of two or more nucleic acids of different origins. However, that conclusion does not logically derive from its own definition.

The word recombinant is a combination of re- (again, back) and combine (COGEM, 2010).

Recombination can mean 'new combinations', as in creating a nucleic acid from parts of different origin and creating a novel sequence over the length of the molecule. However, in molecular biology, as the quote from COGEM also shows, it can mean to 'combine again', to reverse having separated.[43]

For example, during the early stages of developing recombinant DNA techniques particular enzymes (called restriction endonucleases) were used to create smaller DNA molecules from larger ones. This is colloquially referred to as 'cutting' the DNA molecule. A significant challenge was to be able to link 'cut' molecules back together. At one time, to combine the molecules again was the key defining challenge for cloning pieces of DNA *in vitro*[44]. The ability to combine

[43] For example, a history of the origins of recombinant DNA technology describes seminal events leading to creation of isolating short fragments of DNA, their purification and isolation, and then creating longer fragments of DNA from them (including through the use of vectors). "Like Sgaramella, they showed that the two DNA fragments created by the enzyme would spontaneously join, or recombine, with one another" Wright, S. (1986). Recombinant DNA technology and its social transformation, 1972-1982. Osiris *2*, 303-360. This passage demonstrates that researchers saw 'recombine' as to combine again, not just as 'new combinations'.

[44] For example, a quote from a 1976 paper firmly establishes that the recombinant molecule was one that arose because of *in vitro* techniques even though it resulted in a deletion and rejoining of a molecule from the same virus genome. "A novel assay has been developed for *in vitro* genetic recombination of DNA. Substrate and product DNAs are cleaved with a restriction endonuclease and the resulting fragments are separated by electrophoresis in agarose gels. The substrate DNA has been chosen so that the recombination to be studied deletes a segment of DNA…The method provides a convenient and physical, rather than genetic, assessment of the conversion of parental to *recombinant DNA*…In addition, we show that the *in vitro* recombination system completes the *breaking and rejoining steps of recombination".* Emphasis added to Mizuuchi, K., and Nash, H.A. (1976). Restriction assay for integrative recombination of bacteriophage lambda DNA in vitro: requirement for closed circular DNA substrate. Proc Natl Acad Sci USA *73*, 3524-3528. Also "Recombinant DNA technology, which provides new techniques for studying genetics and manipulating genes, was developed over ten years ago. The essence of the technique is easily understood if one thinks of a gene as a piece of a DNA molecule, a chemical entity. DNA can be taken out of cells and tissues and manipulated in a test tube. It can be broken into *pieces which may be joined back together again with the same DNA*, or with DNA from any other living creature". Emphasis added to Sanger, M. (1985). Genetics and the law: a scientist's view. Yale L & Pol'y Rev *315*, 315-335.

again was recognized also as a means to create new combinations which at the time was a motivation for using the new technology (Wright, 1986).

A defining characteristic of 'recombinant' is the ability to combine molecules together again. A derived utility is to combine molecules together in new combinations.

As the history of recombinant DNA technology illustrates, recombination of sequences, in the sense of mixing different sequences together, was not the novelty of recombinant DNA because recombination had already been observed in nature (Lawrence, 1978; Wright, 1986). What made recombinant DNA technology different was the ability of humans to intervene in the process and to choose which pieces of DNA would be combined, unconstrained by the rules operative in nature.

In the 1990s the United States National Institutes of Health (NIH) Guidelines used a defintion that is consistent with molecules joined again and which were created *in vitro*.

recombinant DNA molecules are either (i) molecules which are constructed outside living cells by joining natural or synthetic DNA segments to DNA molecules that can replicate in a living cell; or (ii) DNA molecules that result from the replication of those described in (i) above (emphasis added to Schwartz and Friedman, 1992).

The history of the NIH Guidelines demonstrates that recombinant molecules did not have to be new combinations of sequences. NIH's Recombinant DNA Advisory Committee (RAC) specifically rejected a proposed amendment to the definition of recombinant DNA that would have required the molecule to be a chimera of molecules from different sources:

In 1987 the RAC rejected amending the definition of rDNA [recombinant DNA] to mean '(i) molecules which are constructed outside living cells by joining foreign synthetic DNA segments to DNA molecules that can replicate in a living cell, or (ii) DNA molecules that result from the replication of those described in (i) above.' Recombinant DNA Advisory Committee; Meeting, 51 Fed. Reg. 45,650, 45,651 (1986) (emphasis in original) (Fogleman, 1986-1987).

Furthermore, there is evidence that oligonucleotides—even those of identical composition to natural sequences—are within the scope of Directive 2001/18/EC. EU Directive 2009/41/EC on the contained use of genetically modified micro-organisms is set out similarly to 2001/18/EC. However, in Annex II part A (4) which describes techniques that are exempt from regulation, self-cloning, or using vectors that have a history of safe use, is explicitly exempted.[45]

[45] "Techniques or methods of genetic modification yielding micro-organisms to be excluded from this Directive on condition that they do not involve the use of recombinant-nucleic acid molecules…Self-cloning consisting in the removal of nucleic acid sequences from a cell of an organism which may or may not be followed by reinsertion of all or part of that nucleic acid (or a synthetic equivalent), with or without prior enzymic or mechanical steps, into cells of the same species or into cells of phylogenetically closely related species which can exchange genetic material by natural physiological processes where the resulting micro-organism is unlikely to cause disease to humans, animals or plants. Self-cloning may include the use of recombinant vectors with an extended history of safe use in the particular micro-organisms." http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32009L0041. Access date 8 October 2015.

Hence, Directive 2009/41/EC confirms that self-cloning, which can involve DNA fragments of any size that are identical in sequence, had to be a named exception under the nearly identical qualifier "Techniques or methods of genetic modification…to be excluded from this Directive on condition that they do not involve the use of recombinant-nucleic acid molecules". The exemption is granted under Directive 2009/41/EC because it is specific to work done in only in containment. For such work using recombinant nucleic acid molecules only of the type described in Annex II part A clause 4, a further risk assessment is not required. The specific exemption of procedures that use nucleic acids of the type that may be identical in sequence to natural counterparts in Directive 2009/41/EC reinforces both that such molecules are considered recombinant nucleic acids in the context of Annex 1 B and that the use of such molecules was meant to be captured within the scope of Directive 2001/18/EC.

The history of the NIH Guidelines also demonstrates the interchangability of the concept of recombinant and *in vitro* use. The terms are and were frequently invoked as synonyms. As the journal *Nature* observed—

'Recombinant DNA' means different things to different people. Although it is now almost universally associated with the techniques of in vitro *genetic manipulation…(Lawrence, 1978).*

Recombination in the sense of 'combine again' is a qualitative concept of a 'recombinant nucleic acid molecule', one that is composed of pieces that were again combined. Moreover, it is consistent with the natural/*in vitro* boundary that is used throughout the Directive. Such molecules are thus a subset of those generally *produced by whatever means outside of an organism*.

Moreover, this insight resolves COGEM's uncertainty about how many differences in the order of nucleotides in an oligonucleotide would be enough to make it a recombinant nucleic acid molecule. This is because the difference between a nucleotide and an oligonucleotide is qualitative, not just quantitative, whereas the difference in length of two nucleic acids is quantitative.

A mononucleotide cannot be a sequence. Only molecules of greater than one nucleotide in length have a *sequence of nucleotides*. When embedded into a nucleic acid[46], sequences of as few as two nucleotides can have biological relevance. Those same two nucleotides have no matching biological relevance when they are not part of a nucleic acid.

Conclusion: while the goal of genetic engineering became to make new combinations of DNA molecules, in the development of recombinant DNA technology, to combine previously separated molecules of DNA was to recombine them. That was the seminal biochemical achievement that gave rise to 'recombinant DNA'. Recombinant is and was a common synonym of *in vitro*. Recombinant nucleic acids are a sub-set of those produced by whatever means outside of a cell.

[46] In practice, this may be oligonucleotides composed of around only 20 or so polynucleotides.

Summary

My findings were that:

- OGE is a technique that results in a genetically modified organism as described by Article 2(2) of the Directive.
- Variations of the OGE technique are covered by the descriptions of techniques in Annex 1 A part 1(1) and Annex 1 A part 1(2).
- No variation of the OGE technique is covered by the description of exempted techniques in Annex 1 B.
- As reported by both COGEM and the New Techniques Working Group, a large variety of molecules are described in the scientific literature as oligonucleotides and recombinant nucleic acids and *there is no single scientific view of what these entities are, much less that they are different*. Indeed, the terms are applied to ever-evolving products developed in the scientific community and description varies by when they were first described.
- Mononucleotides (nucleosides, modified or analogues of canonical bases) have a history of use in mutagenesis techniques, significantly predating the Directive. *The use of (mono) nucleotides as mutagens is thus exempted by Article 3 of the Directive*.
- There is evidence that the EU does define oligonucleotides, even those of identical sequence to natural counterparts, as recombinant nucleic acids because in a later Directive they are explicitly described and then exempted for particular uses in containment.
- *There is no evidence that the number of nucleotides in an oligonucleotide was a matter of relevance when the Directive was created, or determinative of what was considered to be an oligonucleotide*.
- *The distinction between all other mutagens and oligonucleotides as mutagens is that only the latter is a nucleic acid. Moreover, the only physical property that all molecules referred to as oligonucleotides or recombinant nucleic acids have in common is that they are composed of more than one nucleotide*. To restate: a nucleic acid is a polymer of nucleotides and never a mononucleotide. A recombinant nucleic acid is also a polymer of nucleotides that has been synthesized or otherwise derived from *in vitro* techniques. A mutagen can be a mononucleotide/nucleoside.
- There is significant variation in how scientists describe a recombinant nucleic acid or an oligonucleotide. It is reasonable to draw the conclusion that these terms were not more certain in 2001 than now. There is no comparable uncertainty in how scientists describe nucleosides/nucleotides.
- Recombinant nucleic acid was a term used synonymously with nucleic acids used *in vitro* already by 1978. It included the concept 'to join again', not exclusively the concept 'to join into new combinations'.
- A characteristic difference between nucleotides and nucleic acids is that the latter can hybridize with another nucleic acid. It is this property that makes OGE 'site-specific' and thus of new commercial interest as a method for changing DNA sequences in organisms. A single nucleoside/mononucleotide can only make changes at random locations.

- It is reasonable to conclude that the use of oligonucleotides for mutagenesis is not excluded in the Directive because it explicitly does not exclude organisms modified through the use of nucleic acids, and *oligonucleotides are nucleic acids*.
- The range of products made using OGE can only be known to be identical to products created through the use of excluded techniques if they are subjected to a risk assessment and this can only be done if they are defined as products covered by GMO legislation.

In my opinion, oligonucleotide-directed genetic engineering (OGE) is not the type of intervention that is captured by the list of mutagenesis techniques exempted from Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms.

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Appendix One

Within science, subgroups of scientists substitute different meanings into the words they use (homonyms), or impose similar meanings on different words (synonyms). This might be sloppy jargon or because to them one of the related meanings is more important than it is to other subgroups using a different word to describe the same thing. Many of the key terms used in Directive 2001/18/EC are such words.

As an example, the word 'homology' has long use predating either the knowledge that genes were made of DNA or the discovery of homologous recombination. In evolutionary biology, homology means relatedness by descent (Fitch, 2000). The wing of a blackbird and a kiwi are homologous even if they have limited overlap of function. In contrast, the wing of a jet airplane and a blackbird are not homologous despite their common function enabling flight. Regardless of how inspired aircraft engineers may have been by bird wings, a jet aircraft did not descend from an ancestor shared by the blackbird.

Homologous has been used extensively in molecular biology to mean DNA 'sequence similarity' (Fitch, 2000). Homology is thus a homonym, a word having two different meanings. Similarity, even identity, of sequence is evidence of homology. It is possible for genes of strikingly different overall sequence to be true homologues of each other, and for genes of strong similarity to be so by chance. The common, but not ubiquitous, use of homology to mean sequence similarity arose from a powerful sub-culture of biology.

Homologous recombination gets its name because it occurs between sequences of high similarity. In the context of organisms that have not been genetically engineered, most often those sequences are homologous; the sequence similarity being a virtue of descent from a common ancestor.

The new technique oligonucleotide-directed genetic engineering relies upon sequence similarity and homologous recombination, at least in the current common manifestations. The biochemistry of homologous recombination works on any two sufficiently similar nucleic acids. Herein ends the similarity with natural processes by which homologous but not identical DNA sequences arise and are used by homologous recombination.

The following list covers terms used in the Directive that are/were common synonyms.

genetic material	genetic information
genetic modification	genetic/genome engineering
heritable material	genetic material
mutagenesis	modification/engineering
produced by whatever means outside of the organism	*in vitro*
recombinant	*in vitro*

Appendix Two

COGEM has attempted to define oligonucleotides to determine if their use would always or sometimes result in a genetically modified organism exempt from regulation.

COGEM defines an oligonucleotide as 'a single-or double-stranded molecule consisting of different nucleotides (or analogues) of DNA and/or RNA with a length up to approximately 120 nucleotides (or base pairs), which may or may not be produced synthetically' (COGEM, 2010).

Oligonucleotides may be used to change DNA sequences, or to modify gene expression through interaction with RNA or protein targets (COGEM, 2006).

Argument 1 – oligonucleotides can cause DNA sequence changes

COGEM believes that the chance that sequence changes are induced in the genome when using oligonucleotides, which interact with RNA or proteins, is insignificantly small. At the same time, COGEM believes that oligonucleotides that interact with DNA can lead to sequence changes (p. 26 COGEM, 2006).

COGEM acknowledges that an intrinsic quality of oligonucleotides is the potential to change DNA sequences if they interact with DNA molecules. However, COGEM argues that where such interactions are not the intended outcome, that is, when they are used to interact with RNA or proteins instead, then the chances of unintended DNA changes are small. Nevertheless, the use of other kinds of DNA molecules, which COGEM calls transgenes, resulting in such changes to the genome would be unambiguously covered by the GMO regulations[47].

Oligonucleotides composed of ribonucleotides might be expected to not change the sequence of bases in a molecule of DNA. However, it is the sequence of nucleotides and not the kind of nucleotides that matters. RNA molecules can bind to DNA, or in some kinds of cells be converted into DNA. They may have the same kind of interactions then with DNA molecules that oligonucleotides intended to interact with DNA can have. Indeed, various combinations of oligonucleotides that include a mix of RNA and DNA nucleotides have been used to cause heritable changes in target genomes (Allyson et al., 1996; Beetham et al., 1999). Although through various controls and modifications of the procedure this possibility can be minimized, a general prevention of the kind of interactions that might result in DNA changes has not been demonstrated.

Therefore, because all oligonucleotides have the potential to cause changes to a DNA sequence, what they are made of does not matter for determining whether use falls within the scope of Directive 2001/18/EC.

Conclusion: there is nothing inherently different between an oligonucleotide and a transgene when it comes to the potential to interact with a molecule of DNA within the cell. Thus if the use of a transgene is defined as creating a genetically modified organism, so must be the use of an oligonucleotide.

[47] "It leaves no doubt that targeted integration of transgenes via 'homologous recombination' and the resulting products are subject to GMO legislation and are obliged to have a license" COGEM (2006). New techniques in plant biotechnology. COGEM advice and report. CGM/061024-02.

Argument 2 – oligonucleotides must be recombinant nucleic acid molecules

Inconsistency in the language of the Directive occurs between Annex 1 A part 1 which uses the phrase 'recombinant nucleic acid techniques' and Annex 1 B which uses the phrase 'recombinant nucleic acid molecules' is interpreted by COGEM to mean that use of recombinant nucleic acid techniques involving nucleic acids that are not recombinant nucleic acids does not result in a regulated organism.

For this reason, COGEM sought to develop a definition of a recombinant nucleic acid molecule to see if oligonucleotides were recombinant nucleic acid molecules. If oligonucleotides were not recombinant nucleic acid molecules, then COGEM believes that the use of OGE would not result in regulated genetically modified organisms.

COGEM regards this definition as suitable for recombinant nucleic acid in the following form:

'Recombinant nucleic acid is a nucleic acid with a nucleotide sequence that does not occur naturally, but is created by combining nucleic acid sequences that do not naturally occur next to each other' (COGEM, 2010).

COGEM explained that a large variety of scientific definitions of recombinant nucleic acid molecules are in use. They all do not exclude oligonucleotides as being recombinant nucleic acids. Disagreement between scientists as to whether oligonucleotides were recombinant nucleic acid molecules was also reported by the European Commission New Techniques Working Group (EC, 2012).

COGEM indicates that any definition of which it can conceive will be arbitrary because there is no singular unifying theme to the kinds of molecules that have been called recombinant nucleic acids. While acknowledging the wide variation of definitions, COGEM nevertheless attempts to settle the matter through a series of assertions, for example, that it "can therefore be said that an oligonucleotide that is substantially similar to - and only a few nucleotides different from - a known sequence should not be considered a recombinant nucleic acid." However, then COGEM notes reservations with this solution, such as anticipated disagreement about what would be "the limits for what is considered similar".

COGEM tries to bring forth some unifying singular feature that would consistently distinguish between mutagens that were and were not recombinant nucleic acids. It appears to not find one and thus adopts a quantitative definition. "In the [19]80s, an 'oligonucleotide' was understood to be a DNA molecule with a length of about 12 to 20 nucleotides. This was what DNA synthesisers could make. Today, that limit has been stretched to approximately 200 nucleotides, and both RNA and DNA molecules can be made. These fragments can, in turn, be linked in vitro to make longer fragments. For scientific purposes, an exact definition is neither relevant nor useful" (COGEM, 2010).

I also have reservations about this approach. Most significantly, I have found no evidence to suggest that those who wrote the Directive were describing recombinant DNA molecules quantitatively. Critically, I found the opposite (see below, section "Clause 1"). In the overall framework of 'recombinant nucleic acid techniques' using nucleic acids 'produced by whatever means outside of a cell',

recombinant DNA molecules were qualitatively different from those which appear naturally, rather than degrees of divergence from what occurs naturally.

Likewise, in the scientific and commercial literature of the time just preceding the Directive there is evidence that scientists were thinking of oligonucleotides as recombinant nucleic acids to be used in recombinant DNA work, even when those oligonucleotides had few or no changes from a corresponding natural sequence[48].

COGEM might have meant that an exact number of nucleotides might not provide a useful definition of an oligonucleotide. With that I can agree. However, it is not clear to me why an exact definition of an oligonucleotide would not be useful.

Conclusion: the use of two different phrases in Annex 1 A and Annex 1 B creates ambiguity in what the Directive is meant to exclude from regulation as a genetically modified organism. However, the reconciliation of the phrases is found in context of Annex 1 A rather than in imagining that a hypothetical quantitative understanding of sequences guided those writing the Directive. Recombinant nucleic acid molecules are 'produced by whatever means outside of a cell' using 'recombinant nucleic acid techniques'.

[48] "An isolated and purified recombinant nucleic acid fragment comprising an oligonucleotide having a contiguous sequence of at least 25 nucleotides in a sequence complementary or identical to a human genomic DNA sequence" Villeponteau, B., Feng, J., Funk, W., and Andrews, W.H. (1996). Mammalian telomerase (Geron Corporation). Patent 5,583,016.
"Chemically synthesized DNA has been used in many recombinant DNA studies. These uses have included the total synthesis and cloning of functional genes, the cloning and expression of natural genes, and editing and changing genes by directed mutation" Itakura, K., and Riggs, A.D. (1980). Chemical DNA synthesis and recombinant DNA studies. Science *209*, 1401-1405.