Biosafety Advisory Council

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Secretariat

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Title: Advice of the Belgian Biosafety Advisory Council on the use of "Targeted Gene Repair" as a strategy to develop novel organisms

Context

On 20 April 2007, the Federal Public Service for Health, Food Chain Safety and the Environment solicited the Biosafety Advisory Council (BAC) for giving a scientific contribution concerning the following question:
Should the "Targeted Gene Repair" technique be considered as a technique of genetic modification yielding genetically modified organisms in the meaning of Directive 2001/18/EC?
This request was addressed to the BAC in the general context of ongoing discussion on this topic at the level of the Competent Authorities dealing with Directive 2001/18/EC.

Procedural aspects

At its meeting of 23 April 2007, the BAC mandated the Division of Biosafety and Biotechnology (SBB) to prepare a draft advice, in close collaboration with one Council member and after consultation of external experts.
On this basis, seven experts were contacted to provide a scientific contribution on this issue. They all had a molecular biology background with specific expertise in plant science, microbiology, microbial biochemistry or human/vertebrate biology. All experts were chosen from the common list of experts drawn up by the Biosafety Advisory Council and the SBB. Five experts answered positively to this request. In addition, the SBB (acting as technical expert) also offered to provide a scientific advice on this issue.

All contributors were asked to assess the targeted gene repair technique as a methodology to develop novel organisms, on the basis of a questionnaire focusing on three aspects:
- How to describe the targeted gene repair technology?
- What the potential applications of this technology are in the context of the development of novel organisms?

- How targeted gene repair technology should be viewed in the context of the GMO definition?

A compilation of the contributions from the external experts and the SBB is provided in Annex I.

Discussion

1. How to describe the targeted gene repair technology?

The "targeted gene repair" (TGR) technology is a generic term covering several techniques and applications. It is referenced in the literature under other names such as targeted nucleotide exchange, chimeraplasty, oligonucleotide-mediated gene editing, chimeric oligonucleotide-dependent mismatch repair, oligonucleotide-mediated gene repair, triplex-forming oligonucleotides induced recombination, oligodeoxynucleotide-directed gene modification, therapeutic nucleic acid repair approach.
All these techniques are based on the site-specific correction or directed mutation (base substitution, addition or deletion) of an episomal or chromosomal target gene after introduction of a synthetic oligonucleotide with homology to that target gene (except for the nucleotide(s) to be changed).

The technology makes use of chimeric RNA/DNA or DNA/DNA molecules (RDOs, 5-35 bp homology domain is sufficient), single stranded DNA oligonucleotides containing phosphorothioate linkages at the 5' and/or 3' end (ODNs, modified ends to protect the molecule against cellular nuclease activity) or triplex-forming oligonucleotides (TFOs).
Introduction of the oligonucleotides can be realised with different techniques that can introduce nucleic acids into cells such as electroporation, lipofection, transfection or particle bombardment (biolistic) with various efficiency, thus without using any vector system. TGR does not involve the introduction of an additional gene (prepared outside the target organism) but modifies the target gene *in situ*.

The observed frequencies of mutagenesis in the treated cells are highly variable (from less than 1% to up to 60%) and appears to be depending on the cell type and on the target locus. The origin of this variability is poorly understood.

There is currently a lack of a detailed knowledge on the mechanisms of action of TGR at the molecular level but according to several papers DNA repair enzymes are involved in this process mainly through the activation of the mismatch repair and/or nucleotide excision repair pathway. The mechanisms are better understood for prokaryotes but, although they seem to be conserved in eukaryotes, not all homologues of prokaryotic repair proteins are known.

2. What are the potential applications of this technology in the context of the development of novel organisms?

The technology is not used for introducing new genes in organisms but for altering natural chromosomal or episomal sequences (genes or control sequences).

In most cases, particularly when applying the technique for therapeutic purposes in human medicine, the aim is to restore a gene defect caused by a point mutation. The introduced genetic change results in somatic gene therapy and remains confined to the treated individual. Also, it merely restores the wild type sequence.
Mutations can also be introduced *in situ* (i.e. site-specific mutations) either to knock-out a deleterious gene (e.g. involved in the production of an antinutritional factor) or to change an amino-acid in the corresponding protein resulting in a protein with possible new properties (e.g. still performing the same function in the organism but insensitive to the action of a specific herbicide) or to induce local changes in expression controlling elements which may lead to subtle changes in the level of gene expression.
One advantage of this technology is that it does not use integrative vectors and thus eliminates the risk of insertional mutagenesis associated with them. It also acts on specific genes and does not introduce foreign sequences in the target cell.
One major drawback is selection, the technology being probably suitable only in cases where corrected cells have a selective advantage or where only a small number of corrected cells generate a therapeutic benefit.

<u>Use of the technology in microorganisms</u>
The technology has been used successfully in yeast mainly as a tool to perform basic research on gene expression and regulation. Apart from that, one can wonder whether the targeted gene repair technology will have larger application in microorganisms. Indeed, the development of genetically modified microorganisms is very easy and efficient through the use of recombinant DNA technology (which allows the use of selectable markers such as antibiotic resistance genes, offering many advantages in terms of selection).
For the time being, microorganisms engineered through this technology have been grown exclusively in contained facilities.

<u>Use of the technology in mammalians</u>
Targeted gene repair directed by chimeric RNA/DNA oligonucleotides has proven successful in animal cells e.g. with globin genes, genes involved in muscular dystrophy, tyrosinase and c-kit gene. Recently, the creation of the first mouse mutant by modification of embryonic stem cells by ss DNA oligonucleotides has been reported. This is a novel approach since this organism does not contain any marker gene. However, typical efficiencies are $<10^4$, leaving mutations too rare to be effectively identified and conditions that can improve the mutagenesis efficiency remain to be found.
The technology seems to offer interesting opportunities for the future in the field of human gene therapy to correct point mutations for instance in inherited disease and cancer therapy. The therapy using oligonucleotides or RNA/DNA chimeras can result in a fraction of cells in which the wild type gene can be restored. Bertoni et al (2005) demonstrated repair of the dystrophin gene in muscle cells in a mouse model (in which dystrophin is knocked out due to a point mutation) for a prolonged period of time.
In many cases, however, there has been a disparity in the levels of gene correction or frequency or reproducibility. The efficacy of delivery of the oligonucleotides into the nucleus, the long-term stability or purity of these molecules, the genetic background of the receiving organism and of the nature of target genes are potential factors that may contribute to this variability. These observations show the need to better understand the underlying mechanisms of gene repair and also to improve animal models.

Use of the technology in plants

Although the technology has been first demonstrated in mammalian cells, preliminary studies published in 1999 showed that TGR technology is applicable to plants and induce target gene mutations. The obtention of herbicide resistant plants through the mutagenesis of the acetolactate synthase gene was demonstrated.

Some major drawback have however been observed such as the difficulty to regenerate transgenic plants or the spontaneous occurrence of somatic mutations which obscure the effect of the TGR approach.

Main applications of this technology are the introduction of mutations that could knock out a gene or restore a gene or modulate the expression of a gene (if a promoter is targeted).

However, considering the fact that the frequency of the repair event is low (but higher as compared to homologous recombination) and that the further selection and regeneration of plants containing the mutation is made difficult due to the absence of selective marker, the TGR technology seems hardly applicable in plants to develop commercial application in the short term.

3. How should targeted gene repair technology be viewed in the context of the GMO definition?

The European definition of a GMO is both technology- and process-oriented. A novel organism will therefore fall under the scope of the GMO Regulation only if it has been developed with the use of certain techniques (such as recombinant nucleic acid techniques), which does not necessarily make sense from the safety point of view. The current exercise of assessing whether the targeted gene repair technology should be considered as a technique of genetic modification yielding genetically modified organisms in the meaning of the European Directives 90/219/EEC and 2001/18/EC is thus both a matter of technical and juridical interpretation of the GMO definition in these Directives. Please notice that the BAC has taken a broad perspective in its advice and has also considered the GMO definition and scope of the 90/219/EEC Directive.

According to the BAC and supported by the views expressed by the Belgian experts involved in the preparation of this advice, it appears that:

- The targeted gene repair technology is not a recombinant nucleic acid technique and does not make use of any vector system.
- The targeted gene repair technology is not a cell fusion or hybridisation technique.
- The targeted gene repair technology can involve micro-injection or micro-encapsulation (in liposomes) to deliver the synthetic oligonucleotide in the cell, although other techniques such as electroporation or particle bombardment are more commonly used. However the BAC is of the opinion that the synthetic oligonucleotides should not be considered as "heritable material prepared outside the organism" in the meaning of Annex IA, Part 1 (2) of Directive 2001/18/EC.
- The targeted gene repair technology does not fall under the techniques referred to in Annex I A, Part 2 of Directive 2001/18/EC or in Annex 1, Part B of Directive 90/219/EEC, which are not considered to result in genetic modification.
- The targeted gene repair technology is a form of mutagenesis. The nucleic acid molecules used in the technology are synthetic oligonucleotides which most of the experts do not consider as being recombinant nucleic acid molecules. These oligonucleotides could even to a certain extend be compared with chemical mutagens.

- Mutations induced by this technology generally involved alteration of 1-2 base pairs in the target site, which is adequate for many applications. However, larger alterations (including deletions or insertions) can occur.
- Organisms developed through the targeted gene repair technology can not be distinguished at molecular level from those developed through "traditional" mutation technology.

The targeted gene repair is a technology used to correct or to introduce specific mutations in defined regions of the genome. According to the experts, the technology differs slightly from the recombinant GM technology due to the fact that the technique does not involve the introduction of an additional gene but modifies the target gene *in situ*, regulated by its own promoter in its natural genetic background.
Theoretically, the targeted gene repair technology should in many cases be more precise than other mutational techniques such as irradiation or chemical treatment, and should lead to less unintended effects. However, given the small sizes of the synthetic oligonucleotides used in this technology, the risk of potential unwanted mutagenesis should not be neglected.
An additional drawback of the method is the lack of means for controlling the specificity of the mutagenesis. While it should be possible to test the efficiency for the mutagenesis of a specific locus, it is much more difficult to test if other unknown non specific loci have been modified at the same time.

The TGR technology is quite old and the first reports were very promising with some spectacular results, but only a few teams have then used the technology and published results. Consequently, it is important to note that organisms produced through this technology have not yet reached the commercial stage (although some patents have already been deposited). Considering this technology in relationship with the GMO regulatory framework is thus for the moment more relevant in the context of the contained use activities (Directive 90/219/EEC amended by Directive 98/81/EC) rather than in the context of environmental releases or commercial applications (Directive 2001/18/EC).

Last but not least, it should be mentioned that this question has also already been investigated by other Member States. In the Netherlands, the COGEM (Commissie Genetische Modificatie) concluded that directed mutagenesis through the use of oligonucleotides was a form of "traditional" mutagenesis and therefore could be excluded from the scope of the GMO regulation.

Conclusions

The Belgian Biosafety Advisory Council:

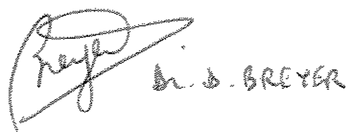
- Notes that the terminology "Targeted Gene Repair" covers a range of different techniques and applications and is therefore not ideal for specific statements or rules.
- Concludes that the technology must be considered as leading to genetic modification.
- Notes that the technology is not used for introducing new genes in organisms but for altering chromosomal or episomal sequences *in situ* in their natural genetic background.
- Notes that the technology does not use integrative vectors and thus the risk of insertional mutagenesis associated with them is eliminated.

- Concludes that the targeted gene repair technology is a form of mutagenesis, a technology which is excluded from the scope of the regulation.
- Notes that the technology makes use of synthetic oligonucleotides which should not be considered as being recombinant nucleic acid molecules.
- Notes that the targeted gene repair technology is in many cases more precise than other mutational techniques such as irradiation or chemical treatment, which should lead to less unintended effects.
- Notes however that the reliability and reproducibility of the technology show a great variability and that further studies are still needed to gain a better knowledge on the mechanisms of action at the molecular level.
- Notes also that the risk of potential side effects in the genome of the recipient cells should not be neglected.
- Notes that organisms developed through the targeted gene repair technology can not be distinguished at molecular level from those developed through "traditional" mutation technology.

Based on the above scientific assessment the Belgian Biosafety Advisory Council:

- Does not share the preliminary opinion of the European Commission of 25 April 2007 that organisms obtained via the targeted gene repair technology systematically fall under the scope of the GMO regulation.
- Considers that there are scientific arguments for not having the targeted gene repair technology falling within the scope of the GMO Directives.
- Supports the setting up of a working group aiming at developing further guidance on this issue and on other related technologies at EU level, and is ready to bring its expertise to the work of this WG.

The conclusions above apply in the context of contained use of GMOs (Directive 90/219/EEC amended by Directive 98/81/EC) as well as in the context of environmental releases or commercial applications of GMOs (Directive 2001/18/EC).



p.o. Prof. D. Reheul
President of the Biosafety Advisory Council

Annex I: Consolidated input from experts who accepted to provide a scientific contribution in the framework of this study (ref. BAC_2006_MI_525)

Annex II: Consolidated list of references (ref. BAC_2006_MI_526)

Bioveiligheidsraad
Conseil de Biosécurité

Brussels, 4 June 2007

Secretariaat
Secrétariat

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Targeted Gene Repair technique as a methodology to develop novel organisms

Annex I: Consolidated input from the experts

The document below is a consolidated list of the answers received from the following experts:
- Prof. Dr. Erik Remaut (Ghent University, Department of Molecular Biomedical Research)
- Prof. Dr. Patrice Soumillion (UCL, Unité de Biochimie)
- Prof. Dr. Lieve Gheysen (Ghent University, Unit Applied Molecular Genetics)
- Dr. A. Brandenburger (ULB, Institute of Molecular Biology and Medicine)
- Dr. J. Van Doorsselaere (KATHO departement verpleegkunde en biotechnologie - HIVB)
- Division of Biosafety and Biotechnology (Scientific Institute of Public Health)

1. Based on your own scientific knowledge and on your survey of the literature, could you provide a brief description of the targeted gene repair technology, focusing on the mechanism(s).

CONTRIBUTION 1

Targeted gene repair uses synthetic oligonucleotides to introduce small nucleotide changes in defined regions of the genome. The vectors are either chimeric, double stranded RNA-DNA or single-stranded DNA molecules that are homologous to the targeted region except for the nucleotide to be changed (base-substitution, addition or deletion). The technique takes advantage of the cellular repair systems, involving mismatch repair, nucleotide excision repair and recombination. The mechanisms are fairly well understood for prokaryotes but, although they seem to be conserved in eukaryotes, not all homologs of prokaryotic repair proteins are known.

CONTRIBUTION 2

Some comments on the definition of TGR.
BROAD: Targeted gene repair relies on the local induction of the cell's endogenous DNA repair mechanisms to attain a gene conversion event within the genome of the targeted cell.
MORE SPECIFIC (in terms of method used): Chimeric RNA/DNA oligonucleotides (RDOs), modified single-stranded oligonucleotides (ssODNs) and triplex-forming oligonucleotides have been developed for site-specific correction of episomal and chromosomal target genes. The gene repair approach relies on specific hybridization

of the oligonucleotides to the target gene generating a mismatch with the targeted point mutation. Restored gene function is anticipated to occur through activation of endogenous repair systems that recognize the created mismatch. (based on, Anderson, J Mol Med, 80, 770-781, 2002; de Semir, Curr Gene Ther, 6, 481-504, 2006.)
REMARK: Another term, which is frequently used in literature is "Targeted Nucleotide Exchange" (TNE). In my view this term more adequately describes the methodology as outlined above.

My answers and comments on the present questionnaire will be based on the above cited definition and methods, with exclusion of e.g. viral vector-based methods.

I am not sufficiently aquainted with the details of the several methods used to review the underlying mechanisms. Although it is generally accepted that mismath repair, as it occurs during normal replication, is also the major pathway for strand-specific elimination of single base mismathes and even small deletions and insertions in TGR, it is at the same time acknowledged that one of the key bottlenecks for exploiting the overall potential of the different targeted gene repair modalities is the lack of a detailed knowledge of their mechanisms of action at the molecular level (de Semir, Curr Gene Ther. 6, 481-504, 2006) .

CONTRIBUTION 3

The "Targeted gene repair" (TGR) technology is variously named in the literature, for example (probably non exhaustive enumeration) : chimeraplasty, oligonucleotide-mediated gene editing, chimeric oligonucleotide-dependent mismatch repair, oligonucleotide-mediated gene repair, triplex-forming oligonucleotides induced recombination, oligodeoxynucleotide-directed gene modification, targeted nucleotide exchange, therapeutic nucleic acid repair approach.
The TGR technology uses synthetic oligonucleotides (ODNs) to introduce small nucleotide changes in defined regions of the genome (deletions, insertions and point mutations). Chimeric RNA/DNA or DNA/DNA molecules and single stranded ODNs containing phosphoro-thioate linkages at the 5' and/or 3' end (protection against cellular nucleases activity) can repair DNA. It should be noticed that both episomal and chromosomal DNA can be modified by this approach.
Introduction of ODNs can be realised with different techniques that can introduce nucleic acids into cells such as electroporation, lipofection or particle bombardment (biolistic) with various efficiency. TGR does not involve the introduction of an additional gene (prepared outside the target organism) but modifies the target gene in situ. The TGR approach relies on the cellular enzymatic gene repair mechanism. However, the underlying mechanisms of gene repair remain to be fully understood.

CONTRIBUTION 4

The targeted gene repair technology is based on the site-specific correction or directed mutation of an episomal or chromosomal target gene after introduction of a chimeric RNA/DNA oligonucleotide or more recently a single-stranded DNA oligonucleotide with homology to that target gene. Introduction can be done with any technique that can efficiently introduce nucleic acids into cells such as electroporation, particle bombardment,… Studies with mutants and with cell extracts have shown the role of host proteins involved in homologous pairing and in DNA repair. The technique does not involve the introduction of an additional gene but modifies the target gene in situ, regulated by its own promotor in its natural genetic background. The technique has been shown to work in yeast and animal cells, and with a lower efficiency in plant cells.

CONTRIBUTION 5

The technology is more than a gene repair technology since it can be used generally for site directed mutagenesis. It consists in introducing, into eucaryotic cells, small chemically synthesized oligonucleotides that are either chimeric DNA/RNA or modified single stranded DNA in order to perform site directed mutagenesis in chromosomal or episomal genomes. The site of mutagenesis is specified in the oligonucleotide sequence but the type of mutation that will be obtained is not. The synthetic oligonucleotide is introduced in the cells by an appropriate method depending on the target cells (biolistic, electroporation, transfection…). The oligonucleotide then goes into the nucleus and mutagenesis occurs via the natural mechanism(s) of DNA repair, using notably the machinery for homologous recombination (RecA type). However, this mechanism is still poorly understood. The observed frequencies of mutagenesis in the treated cells are highly variable (from less than 1% to up to 60%) and appears to be depending on the cell type and on the target locus. The origin of this variability is also poorly understood. Regarding the obtained mutation, it is possible to design the oligonucleotide for a single nucleotide substitution, deletion or insertion but the type of base that will be introduced is not always identical to the one introduced in the oligonucleotide. Again, the origin of this lack of control is not understood.

CONTRIBUTION 6

Several names exist for the "targeted gene repair" technology eg oligonucleotide-mediated gene editing, chimeraplasty, chimeric oligonucleotide-dependent mismatch repair (cdMMR), triplex-forming oligonucleotides induced recombination, and possible other definitions exist or will be used in the future.

Mechanism

Both episomal and chromosomal DNA can be repaired. Chimeric RNA/DNA or DNA/DNA molecules (25 nt homology domain is sufficient, RDOs) and single stranded DNA oligonucleotides containing phosphoro-thioate linkages at the 5' and/or 3' end (so called modified ends to stabilize the molecule, ODNs) can repair DNA (substitutions, frameshifts and (small) deletions or insertions).
Repair of non-transcribed strand is slightly more efficient (in yeast), according to Liu et al (2001) and Yin et al (2005) report that promoter regions can also be modified (using 45 nt end-modified oligonucleotides).
In RNA/DNA chimeras it is the DNA strand that directs repair (Gamper et al, 2000).
In addition, triplex-forming oligonucleotides (TFOs) can also induce gene correction fi Knauert et al (2006) were able to mutate the luciferase reporter gene in mammalian cells. These triplex-forming oligonucleotides can inhibit transcription inhibition but can also direct site-specific DNA damage and thereby induce DNA repair (eg Christensen et al, 2006 and references therein). In principle there is no difference in methodology between the use of oligonucleotides and TOFs.

The complete mechanism is not known but according to several papers DNA repair enzymes are involved in this process. Fi hRad51 (RecA homologue) is thought to mediate three-stranded heteroduplex formation using single-stranded DNA oligonucleotides (Andersen et al, 2002). In the same paper it is suggested that other proteins such as hRad52, hRad54, hRPA and p53 may modulate the formation of heteroduplexes and participate in the activation of the mismatch repair (MMR) and/or nucleotide excision repair (NER) pathway.
In a recent paper, Igoucheva et al (2006) show that some of the enzymes participating in nucleotide excision repair are involved in the gene repair process. This was demonstrated by compairing gene repair efficiencies in cell lines with a specific defective gene in MMR or NER. In this way they identified msh2 and msh3 (two

endonucleases). Cole-Strauss et al (1999) demonstrated that inhibition of msh2 with specific antibodies reduced the cdMMR activity.
A model with the sequence of events at the targeted site is presented in Kmiec (2003).
One can state that the changes in DNA are brought about by endogenous enzymes present in cells.

2. What are the potential applications of this technology in the context of the development of novel organisms ?

CONTRIBUTION 1

Mutations can be corrected in situ e.g. for human gene therapy (although this is not considered a novel organism, certainly not in somatic gene therapy). Mutations can also be introduced in situ (i.e. site-specific mutations) either to knock-out a deleterious gene (e.g. involved in the production of an antinutritional factor) or to change an amino-acid in the corresponding protein resulting in a protein with a different characteristic (e.g. still performing the same function in the organism but insensitive to the action of a specific herbicide).

CONTRIBUTION 2

The technology is not used for introducing new genes in organisms but for altering natural chromosomal or episomal sequences (genes or control sequences). It can be used for inactivating proteins or modulating their properties, and for activating, inactivating or modulating gene expression. The technonoly has been used for developing novel plants but is essentially described with an emphasis on gene therapy applications. Recent reports indicate that the technology is applicable to some stem cells. However, typical efficiencies in mouse embryonic stem cells are $<10^4$, leaving mutations too rare to effectively identify and conditions that can improve the mutagenesis efficiency remains to be found. Overall, the potential for developing novel organisms is obviously high.

CONTRIBUTION 3

This technique is used both for fundamental research to understand the gene repair mechanism and to restore a gene function (gene therapy) or to modify a target gene expression. TGR has been applied to micro-organsisms such as yeast and bacteria, mammalian cells and in plant cells.
Genetic specific modifications have been created by this TGR technology giving “novel organisms” in various experimental settings (e.g. Yeast, bacteria, cell lines, mouse embryonic stem cells, plants). Numerous available publications about the use of TGR show the main potential applications and limits of the technology.
Potential applications of the targeted gene repair technology :
- In micro-organisms (yeast, bacteria). Here the main applications are found in the world of fundamental research and seems to be limited to contained use activities.
- In mammalians : the TGR technology has potential applications in production of Knock-out or mutant mice and in human gene therapy.
Successful application of TGR in mice were published, although the efficiency of the gene correction is low. Obtention of a mutant mice for retinoblastoma gene by modification of embryonic stem cells with ODNs was demonstated. These potential applications remain in the field of fundamental research.

For gene therapy, potential applications are corrections of point mutations for instance in inherited disease and cancer therapy. TGR technology could eliminate the need for integrative vectors and thus the risk of insertional mutagenesis associated with them. The literature reports both experimental success and failure of the TGR technology applied to mammalian cells. These observations show the need to first better understand the underlying mechanisms of gene repair and also to improve animal models.

- In plants

Preliminary studies published in 1999 showed that TGR technology is applicable to plant and induce target gene mutations. The obtention of herbicide resistant plants through the mutagenesis of the acetolactate synthase gene was demonstrated. However it was shown that TGR approach can be obscured by spontaneous somatic mutations observed in plants. An overview of the available literature show that for the moment TGR technology is hardly applicable to plants due mainly to its low efficiency in inducing mutations.

CONTRIBUTION 4

Potential applications are corrections of point mutations for instance in inherited disease. This technology would eliminate the need for integrative vectors and thus the risk of insertional mutagenesis associated with them. It would also only modify existing genes and not introduce foreign sequences in the target cell. The drawback is selection, the technology would probably be suitable only in cases where corrected cells have a selective advantage or where only a small number of corrected cells generate a therapeutic benefit.

CONTRIBUTION 5

I should first like to make a remark on the phrasing 'novel organism'. In my further comments I shall use this connotation as meaning: development of 'novel properties' in a particular organism.

An important consideration here relates to the efficiency with which the targeted nucleotide exchanges can be obtained. Today, the correction efficiencies of mutated bases obtained are in most cases still inadequate for clinical use, and a considerable variation (0.003–60%) in the degree of correction has been reported both in vitro and in vivo. (data from Anderson, J Mol Med, 80, 770-781, 2002).

CONTRIBUTION 6

Under novel organism I would consider organisms that have been genetically modified, a modification that can be in all cells or in a fraction of the cells of the organism and therefore i would assume that the result of genetherapy is a novel organism (an organism that has received DNA that is not present and that has incorporated this DNA in some of the cells).

Use of the technology in yeast

Liu et al (2001) report that genes for which selection can be done (eg introduced marker genes such as GFP or Hygromycine resistance) or for which a clearly different phenotype can be scored (eg slow growth) can be modified in yeast.

In principle it should be possible to use this technology to mutate genes for which a different phenotype can be scored or if some kind of selection (fi on selective media) can be performed. However the creation of genetically modified yeast most of the time involves the introduction of new (recombinant) genes (from foreign organisms).

Also the technology of homologous recombination in yeast (fi to create gene knockouts) is fairly simple and therefore I think that this technology will have only very limited application in this organism. Moreover yeasts are grown in contained use and the presence of selectable markers such as antibiotic resistance genes poses not a problem and the use of selectable markers offers many advantages in terms of practical work.

Use of the technology in mammalians

Several examples of gene repair or knock out (in various cell lines or in vivo animals) eg globin genes (Yin et al, 2005), genes involved in muscular dystrophy (dystrophin genes eg Bertoni and Rando, 2002), tyrosinase and c-kit gene (Alexeev et al, 2002) have been published. According to the literature at least five different research groups have succeeded in using this technology in gene repair experiments. Several papers report on the failure of gene repair or mutation in mammalians eg gauchers disease, apolipoprotein AI, Von Willebrand Factor (vWF, Demeyer et al, 2007 and references therein) and this seems to divide the researchers in this field in two groups (believers and non-believers).

A recent paper by Aarts et al (2006) report the creation of the first mouse mutant by modification of ES cells by ss DNA oligonucleotides. They were able to increase the frequency by transient down regulation (by RNA interference) of genes involved in mismatch repair eg msh2 and used the cells (a pure clone) to create a transgenic mouse containing a mutation in the Retinoblastoma gene.

De Semir and Aran (2006) raise several issues about the consistency and lack of reproducibility. It is mentioned that in order to improve the therapeutic efficacy, the gene repair efficiency should beimproved and that vectors (ss DNA, chimeric RNA/DNA, …) should be modified (eg methylated) to increase gene correction efficiencies. They also warn for PCR artefacts and state that an optimisaton of methods to detect DNA changes should be done and that the mechanism of this process should be studied. The reason why some researchers failed to demonstrate gene repair is for the moment not known but is suggested to be attributed to not-sufficient oligonucleotide quality, inefficient nuclear uptake, the use of different cell lines and different genes, …

To my opinion, this technology will offer opportunities in the future for human gene therapy. The technology is owned by Valigen and I do not know if human clinical trials are underway. The therapy using oligonucleotides or RNA/DNA chimeras can result in a fraction of cells in which the wild type gene can be restored. Fi Bertoni et al (2005) demonstrated repair of the dystrophin gene in muscle cells in a mouse model (in which dystrophin is knocked out due to a point mutation) for a prolonged period of time. Also the modification of stem cells should be possible.
As shown by Aarts et al (2006) it should be possible to mutate any gene in ES cells, select for the mutation using PCR and proceed with the creation of fi transgenic mice. This would be unique since this organism would not contain a marker gene. The question then arisis if this organism is a true GMO since such an organism can arise -in principle- via natural occurring mutations.

Use of the technology in plants

Although the technology has been first demonstrated in mammalian cells this technology has also been used to create transgenic plants. Fi Kochevenko and Willmitzer (2003) demonstrate the creation of herbicide resistant plants through the mutagenesis of the acetolactate synthase gene. This mutation is stably transmitted to the progeny. Zhu et al (1999) and Beetham et al (1999) performed similar experiments (the creation of herbicide resistant cells) but did not regenerate transgenic plants. They also included a mutated GFP that was used as marker.
Hohn and Puchta (1999) mention that in the papers by Zhu et al (1999) and Beetham et al (1999) the correct

changes were seen but that also other changes were noticed, from which it was concluded that the technology is not always as precise. In a paper by Ruiter et al (2003) it is noticed that somatic mutations can occur spontaneously in plant cells and this obscures the effect of chimeraplasty. They regenerated herbicide resistant plants but these regenerants did not have the correct mutation and therefore are the consequence of somaclonal variation in plant cells.

Can this technology be used to create transgenic plants?

For the moment most (if not all) companies active in the field of transgenic plant breeding rely on the method of using Agrobacterium to introduce (foreign or endogenous) genes in plants. The introduction of genes in plants using the TGR technology is not possible. What seems possible using the TGR technology is the introduction of mutations that could knock out a gene or restore a gene or modulate the expression of a gene (if a promoter is targeted).
According to Zhu et al (1999) the frequency is 10^{-4} (which means that 1 cell of 10.000 oligonucleotide receiving cells will convert).
Considering the fact that the frequency of the repair event is low (but higher as compared to homologous recombination) and that after introduction of the mutation in plant cells, a regeneration step is necessary which - without selection- will result in non-mutated (WT) plants and only a small number of plants containing the mutation, it seems not realistic in terms of time and money to perform such an experiment.

3. Among the following statements, which one(s) do you consider relevant to the targeted gene repair technology?

	YES	NO
1. The targeted gene repair technology is a recombinant nucleic acid technique 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.	☐	☒ (6)
2. The targeted gene repair technology is a technique involving the direct introduction into an organism of heritable material prepared outside the organism, by micro-injection, macro-injection or micro-encapsulation.	☒ (4)	☒ (2)
3. The targeted gene repair technology is a cell fusion or hybridisation technique 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.	☐	☒ (6)
4. The targeted gene repair technology is a cell fusion / protoplast fusion of cells which exchange genetic material by known physiological processes or traditional breeding methods.	☐	☒ (6)
5. The targeted gene repair technology is a form of mutagenesis.	☒ (6)	☐
6. The targeted gene repair technology is used exclusively to create single point mutations in the genome.	☒ (1)	☒ (5)
7. The targeted gene repair technology is a form of mutagenesis which makes use of recombinant nucleic acids molecules.	☒ (2)	☒ (5)

8. The targeted gene repair technology is similar to 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.	☒ (1)	☒ (5)

Please justify as far as possible the answers provided above:

CONTRIBUTION 1

- Statement 1: No new combinations; no virus, plasmid or other vector system, no foreign sequences.
- Statement 2: It could in principal be done by micro-injection, but as far as I know this has not been used, probably because it is too labour-intensive.
- Statements 3 and 4: No cell fusion involved.
- Statements 5 and 6: Describe the characteristics of the technique.
- Statement 7: The technique makes use of synthetic nucleic acids, not recombinant.
- Statement 8: There is no removal nor reinsertion involved.

CONTRIBUTION 2

- Statement 1: The technology does not make use of vectorial systems. Synthetic oligonucleotides are introduced in the cells via techniques such electroporation, particules bombardment or lipofection.

The "natural" character of the new genetic material obtained through this technology depends whether one consider the technology itself (which can be seen as artificial engineering) or the genetic material itself (which is the natural genetic background obtained after action of the endogenous gene repair mechanism).

- Statement 2: There are different ways for introducing the oligonucleotides (ssDNA or chimeric RNA/DNA molecules) in the cells. The most commonly used techniques are electroporation (direct uptake by the cells), particules bombardment or lipofection (= micro-encapsulation). However, the injected material (short synthetic oligonucleotides) can not be considered as "heritable material prepared outside the organism".
- Statements 3 and 4: The technology does not involve cell fusion or protoplast fusion.
- Statement 5: The technology is a form of site-directed mutagenesis using appropriately designed gene targeting oligonucleotides.
- Statement 6: Mutations induced by this technology generally involved alteration of 1-2 base pairs in the target site, which is adequate for many applications. However, larger alterations (including deletions or insertions) can occur.
- Statement 7: The answer to this statement depends whether the short ss or ds oligonucleotides should be regarded as "recombinant nucleic acid molecules" or not. Recombinant DNA is generally defined as DNA formed by the artificial combination of several existing DNA strands or by the permanent insertion of foreign DNA. In the targeted gene repair technology, the oligonucleotides are used transiently to induce a point mutation and could be compared to a certain extend to chemical mutagens. This aspect should certainly merit further discussion.
- Statement 8: The targeted gene repair technology is not similar to self-cloning at the process level. However, the oligonucleotide sequence introduced in the cell is a synthetic equivalent of a small part of the endogenous nucleic acid, which makes both techniques very similar at the conceptual level.

CONTRIBUTION 3

- No insertion of an entire nucleic acid molecule.
- The heritable material being the substitution, insertion or deletion of one (or a very small number of) base(s).
- The nucleic acid molecules used in targeted gene repair are synthetic molecules.

CONTRIBUTION 4

- Statement 1: In fact none of the elements cited in this statement apply to the technique of TGR.
- Statement 2: Overall, I agree with this statement. However, I have some remarks on the term 'heritable material'. Clearly the RDO and ssODN molecules are not themselves heritable. Rather, they induce heritable changes once introduced into the targeted genome. Also, 'heritable' here does not in general refer to introduction in the germ line of an organism but remains confined to the cells whose genome was first 'repaired' and to their progeny. For most higher eukaryotes (more specifically for animals and man) the introduced genetic change remains confined to the treated individual. Therefore, the term 'heritable' sensu strictu is restricted to those organisms that may be reproduced asexually (unicellular eukaryotes, some plants).
- Statement 3: Cell fusion is not a part of the mechanism(s) of TGR.
- Statement 4: Same argument as in 3.
- Statement 5: This is entirely correct.
- Statement 6: The technique is not exclusive for single point mutations. The general mechanism(s) of repair should also allow the generation of small deletions and even small insertions.
- Statement 7: NO. The molecules used are nucleic acids which are synthesized in vitro and whose sequence is largely identical, except for one to a few bases, to the target gene. I do not consider these recombinant nucleic acid molecules.
- Statement 8: I agree with this statement with emphasis on 'similar to self cloning' and 'or a synthetic equivalent'.

CONTRIBUTION 5

- Statement 1: The synthetic oligonucleotide is directly introduced in the target cells without the need for a transfer vector.
- Statement 2: But also electroporation, biolistic, transfection.
- Statements 3 and 4: There is no cell fusion involved.
- Statement 5: This is obvious.
- Statement 6: This is true if single base insertion or deletion are considered as point mutations. The technology might be used for creating a double mutation if the sites are separated by a sequence sufficiently short for its compatibility with the oligonucleotide synthesis.
- Statement 7: It's not recombinant nucleic acids molecules but synthetic nucleic acids molecules.
- Statement 8: There is no transfer from one cell to another in this technology.

CONTRIBUTION 6

- Statement 1: No new combinations are made using the TGR technology; the technology can create base

changes, frameshift mutations and (small) insertions or deletions; these changes can occur naturally in organisms.
- Statements 2 and 5: Indeed, ssDNA or chimeric RNA/DNA molecules are introduced in cells via eg injection or encapsulation (eg in several papers lipofectamine is used in transfection experiments) and these molecules will mutate the DNA of the cell in question (the mutation arises through the activity of enzymes involved in DNA repair).
- Statements 3 and 4: Cell fusion is not involved.
- Statement 6: No, also frameshifts and insertions/deletions can be made.
- Statement 7: In the case of RNA/DNA chimeras it is true that recombinant molecules are used; also ssDNA containing phosphorothioate linkages are not natural occuring linkages.
- Statement 8: One can not really say that nucleic acid sequences are removed from a cell.

4. If you are of the opinion that the targeted gene repair does not fit into one of the descriptions given above under 3, could you give an alternative statement best fitting the technology?

CONTRIBUTION 1

- Statement 2: Add "electroporation, biolistic, transfection" at the end of the statement
- Statement 6: The targeted gene repair technology is used exclusively to create single site mutations in the genome, a site being a sequence of less than approximately 50 base pairs.
-Statement 7: The targeted gene repair technology is a form of mutagenesis which makes use of synthetic nucleic acids molecules.

5. Indicate your opinion on whether you consider the targeted gene repair technology (i) to make use of recombinant nucleic acid molecules and (ii) to lead to genetic modification.

CONTRIBUTION 1

Based on the definitions in the directive 2001/18/EC, organisms obtained through this technique could be excluded from the directive as stipulated in annex 1B.

CONTRIBUTION 2

(i) The nucleic acid molecules used in targeted gene repair are synthetic molecules and not recombinant.
(ii) They do lead to genetic modification in the target cell.

CONTRIBUTION 3

(i) NO. The molecules used are nucleic acids which are synthesized in vitro and whose sequence is largely identical, except for one to a few bases, to the target gene. I do not consider these recombinant nucleic acid molecules;

(ii) YES. In addition to repairing a mutated gene back to wild type, the technology can also be used to introduce a mutation (single base change as well as small deletion or insertion).

CONTRIBUTION 4

(i) The technology makes use of synthetic nucleic acids without the need of a transfer vector, so this nucleic acid has not been replicated in vivo before entering the target cells and, in this sense, it has no recombinant nature. However, when this synthetic oligonucleotide reaches its genome target, the repair mechanism that leads to mutagenesis is a form of recombination between the cellular and the synthetic DNA. The obtained "transfected" cell can therefore be defined as a recombinant product.
(ii) Since the technology affords nucleotide substitution, insertion or deletion in a genome, it must be considered as a technology leading to genetic modification.

CONTRIBUTION 5

- The answer to point (i) depends whether the short ss or ds oligonucleotides should be regarded as "recombinant nucleic acid molecules" or not. Recombinant DNA is generally defined as DNA formed by the artificial combination of several existing DNA strands or by the permanent insertion of foreign DNA. In the targeted gene repair technology, the oligonucleotides are used transiently to induce a point mutation and could be compared to a certain extend to chemical mutagens. This aspect should certainly merit further discussion.
- Regarding point (ii), the targeted gene repair technology is used mainly to create genetic mutations (which of course lead to genetic modifications). In terms of objectives, it can be considered as being more closed to mutagenesis (or even self-cloning) than to vectorial technologies. It is even more precise than chemical mutagenesis that very often hits far more target sites in the genome.

CONTRIBUTION 6

The technology makes use of recombinant nucleic acid molecules eg the RNA/DNA chimeras (molecules that do not occur naturally). The result is genetic modification but not as "genetic modification" is mostly done eg introduction of new recombinant genes using a selectable marker eg antibiotic resistant gene and therefore to my opinion gene restoration or gene mutation would be a better terminology.

6. Please provide a list of references (scientific papers, internet sites, patents, ...) relevant in the context of the current questionnaire.

See a consolidated list of all references given by the experts in Annex II.

7. Please indicate any other comments you have regarding the targeted gene repair technology and its potential use as a methodology to develop novel organisms.

CONTRIBUTION 1

Targeted gene repair is a promising methodology for the development of novel organisms. However research is still needed to fully understand the mechanisms before it can be used in clinical trials. This will in turn probably help increase the efficiency and reproducibility of the approach.

CONTRIBUTION 2

Of course it can be argued that this is not a 'natural' technique because it uses chemically synthesized DNA that is introduced in the cell by chemical or physical means, but irradiation has far more impact on the genome than this method. The genetic changes introduced by this technique are much less than those introduced by mutation breeding or induced translocation breeding (involving irradiation). Whether you stick to the definitions of GM or look at the consequences of this technique, the conclusion is similar: this technique differs from GM-technology. Cisgenesis is another technique that could be discussed in the future (Jacobsen and Schouten 2007, Trends in Biotech. 25 p. 220).

CONTRIBUTION 3

When mining the literature, I was surprised that, while this technology was quite old and the first reports were very promising with some spectacular results, only a few teams have then used the technology and published results. The major problem is probably the lack of means for controlling the specificity of the method. Indeed, while it is possible to test the efficiency of the technology for the mutagenesis of a specific locus and to create specifically modified organisms, it is most probably not possible to test if other unknown non specific locus have been simultaneously modified. Given the small sizes of the synthetic oligonucleotides used in this methodology, the risk of this potential uncontrolled mutagenesis is probably high. Nevertheless, even in this case, the methodology appears to have a large potential for the development of novel organisms. Moreover, this potential is increasing with the increasing number of sequenced genomes and the progresses of our knowledge in protein structure-function relationships and gene expression control.

CONTRIBUTION 4

In most cases, particularly when applying the technique for therapeutic purposes in human medicine, the aim is to restore a gene defect caused by a point mutation. The introduced genetic change results in somatic gene therapy and remains confined to the treated individual. Also, it merely restores the wild type sequence. For these two reasons, such an approach cannot be considered as giving rise to a novel organism.
Organisms with novel properties may, however, be produced by applying the targeted nucleotide exchange technique. The method allows the conversion of single or even a few nucleotides in any given gene or gene expression controlling element. In this way (i) stop codons may be created in existing genes, resulting in a disruption of their function, (ii) codon changes may be introduced, resulting in genes encoding proteins with altered amino acid composition and possible new properties, (iii) local changes in expression controlling

elements may result in subtle changes of the level of gene expression, which may result in pleitopic effects in the affected individual. For most higher eukaryotes (more specifically for animals and man) the introduced genetic change remains confined to the treated individual, i.e. a 'novel organism' has in fact not been generated. This would, however, be the case when it becomes feasible to introduce the above mentioned alterations in the germ line of an organism. Today, this 'generation of a novel organism' can already be envisaged for those organisms that may be reproduced asexually (unicellular eukaryotes, some plants).

CONTRIBUTION 5

- This technology has been used since many years in the laboratory. It is important to note that organisms produced through this technology have not reached yet the commercial stage. Considering this technology in relationship with the GMO regulatory framework is thus for the moment more relevant in the context of the contained use activities (Directive 90/219/EEC amended by Directive 98/81/EC) rather than in the context of environmental releases or commercial applications (Directive 2001/18/EC).
- The terms "Targeted Gene Repair" covers a range of different techniques and application which makes it difficult to issue general statements.
- The targeted gene repair technology is in many cases far more precise and direct than other mutational techniques such as irradiation or chemical treatment, which should lead to less unexpected effects. This is an important point given to the fact that "traditional" mutation technology is exempted from the scope of the GMO regulation.
- Organisms developed through the targeted gene repair technology can not be distinguished at molecular level from those developed through "traditional" mutation technology.

Bioveiligheidsraad
Conseil de Biosécurité

Brussels, 4 June 2007

Secretariaat
Secrétariat

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Targeted Gene Repair technique as a methodology to develop novel organisms

Annex II: List of references

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