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Safety in the Contained Use and Release of Transgenic Animals and Recombinant Proteins

Consultant. **Don Powell**
Bookstop Off: M. Tados, IACT/TDA/TP/BGE

Cell Determination Laboratory,

Department of Development and Signalling,

AFRC Institute of Animal Physiology & Genetics Research,

Babraham, Cambridge CB2 4AT, UK

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INTRODUCTION

In one sense, genetic manipulation of animals has been practised for thousands of years in the breeding of domestic species from cats to cows. It has taken but a few hundred years for humans to derive, from a common wolf stock, dogs as diverse as the Pekinese and the Great Dane. In these breeding programmes, whether 'scientifically' designed or not, animals displaying the 'desirable' traits are allowed to breed and the 'undesirable' traits are bred out. This type of programming, upon which all classical breeding experiments are founded, relies on chromosome recombination and the random assortment of chromosomes to bring together desirable traits; this is a lengthy and costly business. Modern technologies in animal breeding represent a dramatic change in which nuclear transfer, cloning, sexing and transgenic biology may generate dramatic shifts in the phenotypes of animals. These changes may bring new benefits, but what problems do they pose? In this chapter, I discuss the nature of transgenic animals and recombinant proteins within the framework of the impact of these technologies on the environment. I shall discuss existing regulations, especially of the USA and the UK, which have taken different approaches to the same problems. It is interesting to note that, while the USDA has approved over 300 releases of transgenic plants, only a single contained release of a transgenic animal (carp) has been fully implemented. The reasons, amongst others, for this difference relate to complexity of the traits being engineered into animals and to public perception.

Terms of reference

The remit begs the question: what defines a genetically manipulated organism (GMO), transgenic animal or a recombinant protein? A transgenic animal is a simple concept; in essence, it is any animal whose genome contains DNA sequences (a transgene) not found in

either parent. In research experiments this DNA may direct the synthesis of a functional protein, such as growth hormone or α_1-antitrypsin; it may direct synthesis of a marker proteins, such as β-galactosidase; it may direct synthesis of no protein, serving as a DNA marker in the genome. Each of these is transgenic, although only the first category is designed to alter the physiology of the animal. This is the category on which I shall concentrate. The transgenic animal may be derived by one of several routes (see Production of Recombinant Genomes, below), each of which will convey different characteristics on the organism.

Recombinant proteins are derived from DNA that has been manipulated *in vitro* (rDNA), and may be produced by joining together 'natural' gene sequences or by deletion of gene sequences or by addition of synthesized DNA sequences. A narrow definition of a recombinant protein is the one that contains sequences that differ from those found in nature. A broader definition, and one that is usually applied, is that a recombinant protein is one synthesized from an exogenous gene or transgene, whether in *Escherichia coli*, yeast, mammalian cells or a transgenic animal. I argue in the following section that this definition is an unfortunate one for those concerned with safety.

Are recombinant genes and proteins special?

By their means of production, recombinant genomes and genes are special. They are founded on technologies that are only a decade or so old. But what consequences does this have for their safety? The safety of any product, whether biological, chemical or physical, is defined by its *behaviour*, or *properties* not its method of production[1]. The safety of an automobile is defined by its behaviour in safety tests and not by whether it is made by hand or made on a production line. In this sense, GMOs do not from a special category because

of their means of production and it is widely accepted that this means of production is not associated with special risk categories. If the behaviour or properties of a recombinant product (gene or protein) differ from those of natural products, then it is important to assess the implications of that novel behaviour.

For example, a transgene may be less stable than an endogenous gene. This is an example of a property that must be addressed in assessment. However, it is the biological properties of the novel genotype that determine behaviour in the environment. This applies as much to novel 'natural' genomes as to recombinant genomes. The release of the rabbit, an entirely novel but also entirely 'natural' genome, in Australia has caused far more damaging and wide-ranging consequences that most planned releases of transgenic animals will.

In a similar vein, recombinant proteins do not necessarily form a special risk category because of their means of production. Although we may use genetic manipulation to produce a protein with properties that are not found in nature, determination of hazards associated with that protein will largely follow the same guidelines used to assess any novel food, drug or industrial component. The environmental consequences of recombinant genomes thus do not alter in kind from the consequences of natural but exogenous genomes.

Applications of recombinant genomes

Transgenic animals

In principle, transgenesis offers a means to generate either subtle or dramatic changes in phenotypes.

1. It may induce the 'improvement' of an existing trait in an animal. In these experiments, transgenesis is used to circumvent or to improve upon the alternative of

existing breeding programmes designed to fix a desirable trait in an already valuable lines. The transgenic approach might thus be used to make a livestock species grow faster[2], to alter yield of milk[3], or to transfer a disease-resistant property from one strain to another[4].

2. It may confer entirely new properties on a species. In this approach, a foreign protein might be produced in a transgenic animal (for example pharmaceutical proteins in the milk of ungulates[5-7], or it may confer new disease-resistant properties[8,9], or alter the immune system to generate new histocompatibility antigens or new antibodies[10], or confer new digestive capabilities to improve the calorific use of less digestible feedstuffs[11].

The range of potential applications thus extends from animal husbandry to pharmaceutical industry. Some examples of such animals are listed in Table 1.

Recombinant proteins

The range of application of recombinant proteins is as wide as the current use of 'natural' proteins. Any industrial process, from brewing or cheese-making to production of pharmaceuticals, may, potentially, be modified by the use either of recombinant proteins or of microorganisms producing such proteins[18,19]. Indeed, in the UK genetically engineered rennin is used in the production of cheese and engineered yeast in production of bread and in the pilot-scale production of beer. By 1991 the US Food and Drug Administration (FDA) had approved more than 12 therapeutic agents and vaccines and had permitted clinical testing of some 800 more derived from rDNA[1]. Recombinant proteins have thus become well established in industrial and clinical life, while release of transgenic animals (and plants) has proceeded much more cautiously. Such technologies of recombinant protein production may

be well adapted to the agriculture and needs of the development world. In particular, the production of recombinant proteins in the developing nations may be in part based on baculovirus expression systems. Baculoviruses, whose natural hosts are insects have been manipulated to produce vector systems that will efficiently express foreign proteins in insect larvae[20]. Under this area of major value to developing countries would also be included the development of recombinant vaccines, which have the potential to reduce many diseases of humans and animals (see below).

Production of recombinant genomes

Transgenic animals

The history of making transgenic animals is a little over 10 years old[21]. During that time, several mechanisms by which DNA can be incorporated into a animal have been developed (Table 2). In mammalian embryos, microinjection of DNA into the pronucleus of the fertilized egg (usually with the larger, male pronucleus) is used by many groups and has been used to generate the vast majority of transgenic mouse lines. Although largely reliable, the site of integration is random, the number of copies of DNA integrated can be unpredictable (though this can be targeted towards zero to two or three copies by using low concentrations of DNA) and a proportion of animals will be mosaic (not all cells contain the transgene). However, breeding programmes to establish the transgenic line can be used to select individuals containing only one copy, to determine that expression is at appropriate levels and to establish that the homozygous transgene is stable in the host genome. Once these parameters are determined, the transgene will then behave as any 'normal', endogenous, host gene.

Embryonic stem (ES) cells are cells derived from the early embryo that can be grown

in culture and then returned to a recipient embryo where their progeny will contribute to all the tissues of the developing organism[28]. During this culture period, foreign DNA can be introduced into the cells using simple methods and the expression and stability of the transgene can be verified. The major advantage of ES cells is that very subtle modifications can be introduced into the host genome[29]. Constructs can be designed that are homologous with a host gene but contain an interruption or modification of that gene sequence. In rare cases, the DNA transfection *in vitro* will lead to integration into the endogenous gene, based on the homology between the transgene and the endogenous gene. In this way, host gene sequences can be altered at any level of subtlety, from single base-pair changes to deletion of much of the protein-coding region[30]. This sophisticated technology is unique to mammalian species and principally, as yet, to rodents. Much effort and considerable expense around the world is devoted to obtaining reliable ES cell cultures from domestic species in order to realize these invaluable techniques in agriculturally important species[22].

Retroviral vectors have held much promise since their development in the early 1980s[31]. Retroviruses are RNA viruses that make a DNA copy of themselves during replication; this DNA copy then integrates into the genome of the host cell. Using recombinant DNA technology, the viral genes can be removed leaving only those functions required for integration of DNA into the host genome. Foreign DNA can then be added to these viral sequences and transferred to recipient cells using a 'helper' cell line that provides the deleted viral functions. Because of problems with stable expression and fears about the safety of viral vectors, such experiments have been limited in animal biotechnology. However, they have been used to produce transgenic poultry[9,17] (in which retroviruses were first discovered), and they have also formed the only tool used to date for human gene therapy[32] and may be resurrected for livestock species.

Sperm-mediated DNA transformation is the technique of potentially widest application but is also that with the most unreliable history[25-27]. Because it is a very simple technique (it basically consists of mixing DNA with sperm and then performing *in vitro* fertilization) it could be used in laboratories throughout the world. However, its low reliability, coupled with questions about the stability of transgenes, means it remains at the research stage.

Other techniques, such as electroporation, have been tried and found application in some areas in which the more 'conventional' techniques have proved unreliable[33].

Recombinant proteins

There are many therapeutic proteins used throughout human and animal medicine. The majority of these are derived from animal or human tissue. It has been possible to purify exceedingly low concentrations of, for example, growth hormone from porcine or human pituitaries, or Factor IX from human blood to acceptable levels of purity. Recent concern has focused on the possibility that such human or animal tissues can be contaminated with slow viruses or retroviruses. Fortunately such concerns were developed contemporaneously with recombinant DNA technologies and a variety of synthetic systems has become available[34]. Table 3 lists a selection of those currently in use as well as some that are in development. Broadly, prokaryotic systems can be tuned to high efficiency, but may be deficient in some of the processing steps commonly carried out in eukaryotic cells (glycosylation, protein cleavage). The problem of processing is not unique to prokaryotic cells and a great deal of effort has been devoted to obtaining efficient modification of proteins in eukaryotic systems[6].

Live attenuated vaccines are commonly used for prophylaxis in human and animal populations: indeed, such use can be regarded as the largest planned introduction of GMOs,

with an enormously successful history[1]. Genetic manipulation offers one way of refining this course of disease eradication or control. In Europe, rabies is commonly carried in the fox population. In campaigns using attenuated virus strains, it was found that the proposed vaccine remained pathogenic for rodents (and the raccoon, a major vector in the US) and could revert to virulence. In a major environmental test, a recombinant vaccinia-rabies surface glycoprotein vaccine appeared to be highly effective in eradicating rabies from the fox population in a 2200 km^2 region of southern Belgium[41]. This programme followed the recommendations of WHO for trials of oral rabies vaccines, which had reviewed such field trials and made recommendations for future research[42]. Recently, a vaccine to protect poultry against Newcastle disease virus, based on a herpes virus of turkeys vector has been developed[43], and such developments will increase as understanding of viral vector biology and of virus spread accumulates.

EXISTING REGULATIONS

History of regulations

As many countries practice genetic manipulations, so there are sets of regulations. In 1991 UNIDO published its *Voluntary Code of Conduct for the Release of Organisms into the Environment*[44], one of the intentions of which was to harmonize global codes of conduct. In the UK, in addition to two European Community (EC) directives framed under Directoire-General (DG) XI[45,46], soon to be subsumed under DG III and DG VI, and the Genetic Manipulation Regulations, with 11 Notes of Guidance, there are nine Acts of Parliament on the production and release of GMOs. Australia labours under the weight of 23 regulations. Many countries have attempted to use existing legislation (such as for regulation of animal health, the environment, foods or chemicals) to cover many aspects of use of GMOs and

imposed additional regulations to supplement these. In many ways this is a reasonable course of action. As I propounded above, GMOs, defined by their product, will in most cases represent no risk different in kind to other novel products. If one accepts this concept, it may be that existing legislation will cover the safety aspects at issue, be they release of organism, safety of the individual, safety of the food or of the drug product.

Regulation has fallen into the two major camps of process *versus* product. Many countries have chosen to regulate genetic manipulation at the laboratory level and have subsequently extended this regulatory oversight to large-scale use and release. This has largely been the experience in the UK, where assessment (see below) is applied specifically to GMOs. By contrast, the USA has developed, after considerable and still rumbling debate, a risk-based oversight of environmental experiments that addresses risk on the examination of the nature of the organism and its intended release site[1,47,48].

Safety in the production of GMOs

The area where legislation must be most carefully studied is in the production and assessment of GMOs, for it is at this stage that the organism is novel and uncharacterized. In the UK, the regulations, together with the associated Notes of Guidance were originally framed in the 1970s and early 1980s. With a greater perception of the risks of laboratory-scale work, many of the original Guidelines were modified[49]. Nevertheless, these regulations still maintain that if there is a risk of expression of the DNA then the categorization is raised to a higher level. Similarly, if the protein product has toxic properties than a higher level still may be required. Clearly then, a fundamental understanding of the DNA sequences being manipulated is crucial to properly assessing, and therefore perhaps relaxing, the safety criteria imposed. Using an arbitrary scale of factors that indicate 'access' to the protein (the

likelihood that the GMO or its DNA will enter the human and survive), 'expression' of the protein (defined by whether the site in the vector is designed tc make the cloned protein), and 'damage' by the protein (a measure of the risk to health of the worker) give a combinational assessment of the relative risk; this then allows categorization of the experiment into one of four classes. This categorization is based on work with non-recombinant pathogenic organisms.

Two of the Notes (1 and 5) govern the use of oncogenic sequences and viral vectors. Because of the possibility of human infection, restriction on the use of such systems is tighter. However, it should be noted that no human infection has clearly been demonstrated to be due to laboratory work with a GMO.

CONTAINED USE AND RELEASE

Contained use of recombinant genomes

For many transgenic species, containment at the research level is already practised as part of good animal husbandry. Special care will normally be taken to ensure no access to research animals by their wild-type relatives. Similarly care will be taken, especially since each of the animals is extremely 'valuable' at this stage, to ensure that no escape to the environment is possible. Similarly, access of common pests to the research site should be prevented using conventional pest control methods. Such restrictions apply to all transgenic species. Those animals, for example insects or fish, that are not readily contained should be held under particularly careful conditions. Assessment of the research on transgenic carp at the Alabama Agricultural Experiment Station (Auburn, AL, USA) found that there was no significant impact of release of the modified fish into a contained facility [50]. In their 67-page assessment, the Research Station considered five alternative approaches to containment

and took into consideration such aspects as flooding leading to release of the carp. A similar proposal for contained release of transgenic catfish was subject to equally or even more rigorous assessment[51].

Release of recombinant genomes

Potential hazards of recombinant genomes

There is a history of several hundred years of release of novel genotypes into the environment. In the area of biological pest control, hundreds of organisms have been released into new locations in an attempt to eradicate pests of agronomically important crops[52]. One of the earliest successful releases was the vedalia ladybeetle to control cottony-cushion scale in California in 1988-9. A long search of Australian ladybeetles finally selected a suitable parasite. More recently, a large-scale attempt to control the cassava mealybug in Africa has been undertaken. In this case the predator is a South American organism *Epidinocasis lopezi*. The history of such releases has seen many great successes. There have also been failures, most often when the relationship between predator and prey or host and parasite were understood only poorly. The major lesson from these experiments (many of which were carried out over the last hundred years) is that a sound understanding of the ecology of the new location is essential. The ecology of the host crop (be it plant or animal) and its relationship with the pest are most important. An assessment of the validity of the new control agent in contained facilities is also crucial. Steps such as these have proven essential if the risks are to be minimized. The introduction of the rabbit into Australia, where there was no effective competition for it, is an example of a particularly misguided attempt, not to control a pest, but simply to provide sport.

The successful introductions (as well as the unsuccessful introductions) emphasize the

requirement for a background understanding of such ecosystems. The introduction of an entirely novel genotype into the environment will, in most cases, have much more extreme consequences than the introduction of a transgenic organism. Information about hazards arising from the release of transgenic animals can be sought under several categories (Table 4). Through laboratory and research site assessment, many of the characteristics of the transgenic organism can be compared with homologous, non-transgenic individuals: its feeding behaviour, its sexual behaviour and aggressive behaviour and its movement can be assessed under containment. It is clear that some of these hazards will be of minor relevance to some experiments; however the regulations of most nations require that they be considered.

Safety and assessment committees

As far as possible, the proponents of a release should ensure that they exercise the maximum amount of care in planning their intended release. There is thus a need for an expert body with a range of interests to examine releases that are sufficiently novel to cause concern, whether these releases are of transgenic or non-transgenic species. Their role is to bring their own expertise to bear on the question of release. In the UK, such a committee would include many of those who have planned the release as well as those in control of the research establishment, since the responsibility for safety lies with them[53]. In addition it might be important to use skills of experts in:

- genetics
- ecology
- safety
- molecular biology

- botany
- entomology
- environmental health

In the USA, it is normal to include an ethicist (from a background of philosophy or religion) on such a committee.

The task of the committee is to investigate the security of the release site and to use their skills and to apply lateral thinking in order to envisage risks and to attempt to estimate their significance and consequence(s). It is important that at an early stage the public is made aware of the intended release. Openness in discussing proposed releases is an important part of informing the public of perceived hazards, of methods of estimating their likelihood and persuading them that all reasonable precautions have been taken.

Safety and risk evaluation

The procedure of preparing for release in the UK is covered by the GENHAZ system[51]. This is based on the chemical industries' assessment scheme and is designed to force the assessors to examine consequences of the release, no matter how unlikely and, more important, to force them to generate as precise outcomes as possible (Table 5). It is discussed here as an example of a common mechanism of directing assessment of environmental consequences of release of a GMO. The essential components are a set of keywords and guide word designed to help frame these questions. One such guide word is 'WHERE ELSE': it may be needed to ask: what happens if the DNA is detected somewhere other than at its original integration site; what happens if expression occurs in tissues other than the intended site; what happens if the organism is found at a location other than its intended release site. By considering these combinations of key words and guide words in

the background of a wide range of understanding, the large majority of possible (as well as unlikely) outcomes must be answered.

Clearly the implications for release of a GMO are more severe if that organism cannot readily be recovered. Transgenic fish are a case in point. Growth hormone and cold-tolerance genes have been introduced into lines of fish. It appears that both experiments have been successful at the laboratory level: transgenic fish containing GH genes have been produced that grow larger than their non-transgenic relatives; similarly more cold-tolerant fish have been generated using the antifreeze gene.

At an intuitive level, it might be thought that both such populations could pose a threat to natural ecosystems, the larger fish feeding more aggressively, the cold-tolerant fish displacing natural species from cooler waters. In the contained releases envisaged, several factors argue against this[50,51].

- Escape of any fish is an unlikely event, and could not involve the large numbers required to establish a new genotype in the environment
- The mirror carp chosen for the experiments is less hardy than the naturally occurring domestic carp - it is at a selective disadvantage
- Small numbers of escaping fish are unlikely to become established (fixed) in the environment; they may well be geographically isolated and their breeding patterns may differ from non-transgenic relatives
- The transgene is unlikely to become fixed in the natural population unless it is under positive selection pressure
- Even if the transgenic fish became fixed in the local environment, it would still be subject to the biological control (disease, predators, food shortage) that affect the natural carp

In a well-designed release, therefore, it is possible to identify not only artificial barriers to ecological imbalance but also the (perhaps more important) natural biological barriers. Because the GMO is, most often, a weaker, domesticated strain of natural relatives, the types of barrier that might operate can, if necessary, be studied in some detail.

The risk assessment should be examined by a competent body that is independent of the research group. Invariably national review bodies will be established, but it is important now, and will become more important in the future, that such natural bodies communication with one another (see below). This body will have many of the expertises of the committee preparing the release assessment and, in addition, competent persons to examine national regulations and laws.

Impact after release

A controlled release allows the time to determine properties of the GMO through several seasons of breeding. Laboratory experiments will reveal many of the alterations that may have occurred in behaviour of the GMO, but it is in more natural ecological environments that behaviour may be fully assessed. If it is felt to be necessary, contained release allows the study of behaviour in competition with natural species. Many of these concepts would be part of normal good agricultural practice. New strains of domestic animals or fish would normally be tested before being used on a large scale to replace a current organism. Such assessment, for example of increased milk production through non-transgenic means in cattle, would include several generations to assess the stability of the phenotype and to determine any deleterious effects on the organism. These steps are essential in all classical breeding programmes before an acceptable breeding line can be established. In a similar way, a controlled release of GMOs that are sufficiently novel, or

sufficiently distinct in their properties from their parent animals, provides the opportunity to investigate the impact of these differences.

The classical common sense of breeding programmes, then, applies as much to new strains produced by the interbreeding of selected animals as it does to GMOs. In aquaculture, the introduction of a novel food species requires as much attention and care as the use of a genetically modified fish stock.

CONCLUSIONS

Biology, common sense and release

Throughout this chapter several themes have been reiterated. The need to recognise the essential similarity between classical and biotechnological genetic manipulation should be a guiding force. When considering the risks attached to such manipulated organisms, it should be the biological principle that it is properties of the animal or protein that dictate the hazards, not the means by which that animal or protein was derived that is of prime importance. Acceptance of this argument may well mean that existing legislation can be used to regulate recombinant DNA releases. The products of genetic manipulation can, in most cases, be compared with novel foods, proteins or crops produced by traditional means *except we understand far more clearly the genetic changes in the GMO than we do in the 'traditional' product.* Most countries have legislation that covers hazardous or testing of pathogenic or exotic organisms or unapproved drugs or chemicals. Such statutes may well provide sufficient regulatory oversight. The scale of this problem should not be underestimated. Each year approximately 11 potential pests enter the USA through all modes of unintentional transfer and, of these, seven are likely to be injurious[52]. There is also the human dimension: in 1986, the USDA intercepted, at ports of entry, nearly 50 000

attempted introductions of exotic organisms. It is important to remember that some 'traditional' practises in agriculture or industry are less sophisticated and may be more hazardous than biotechnological solutions to the same problem.

Against this must be set the public perception of genetic manipulation. It is important that the public - the consumer - is aware that the type of change wrought by genetic manipulation is often more subtle, more predictable and more defined than the changes produced by classical means. Education about the background to traditional and recombinant methods should help to reassure the consumer.

However, the arguments about regulation have recently increased. In the US, the experience gained with the release or movement of 1000 genetically modified plants has led to a proposal that regulatory "...oversight should be more commensurate with scientific indications of potential risk...comparable with that historically applied to conventional plants"[54]. However, confusion still exists over the US FDA's policy and Jeremy Rifkind's Pure Food Campaign pressure group is foremost among critics[55]. Criticism of the European regulations was raised in 1989[1]: the debate about the need for stringent regulations and the influence they have on investment has raged since[56-58]. It appears now that many releases will fall under the influence of the new, less process-based regulations (DG III and DG VI), rather than those originally proposed. Nevertheless, only six of twelve member states have even partially ratified these proposals.

It is important to appreciate that decisions on the level of regulation are very significant factors in industrial view of investment opportunities. Bayer AG of Germany and NOVO Industry of Denmark have both established major research and development facilities outside their home centres in part as a reaction to the vigorous regulations imposed. Aspects of social, economic and technological implications of biotechnology for the developing world have been discussed recently[59].

Framing legislation

With this social and economic constraints in mind, it may be valuable to ask oneself how to frame legislation and what is the need for new legislation (Table 6). The starting point is to examine existing legislation, in order to determine whether most or even all aspects of release of GMOs can be covered. All countries hold that safety and rick assessment of production of GMOs forms a special category requiring legislation. This is based on the view that in many cloning experiments the precise nature of products generated cannot be predicted. In the release and use of transgenic animals or proteins many countries have made use of the existing (perhaps modified) legislation. In the UK such legislation includes the Environmental Protection Act and the Food and Environment Protection Act; in the USA, the Coordinated Framework for the Regulation of Biotechnology establishes that existing laws (such as the Animal Quarantine Laws) are sufficient to regulate the products of biotechnology.

In the absence of appropriate existing legislation, then the discussions above, together guidance supplied by the *Voluntary Code of Conduct for the Release of Organisms into the Environment* provide a framework for general principles.

International resources

There are several bodies that seek to harmonize and integrate international biosafety, for this is inevitably a transnational concern. UNIDO was instrumental in bringing together a panel to draw up the *Voluntary Code of Conduct for the Release of Organisms into the Environment*[44]. It has established research centres, under the ICGEB, in Trieste and New Delhi, designed to assist technology transfer between the industrial and the developing world. ICGEB, which will shortly become autonomous, is 'owned' by its member states and with its flexibility is responsive to the changing needs of those countries. It also maintains a

computer network, ICGEBnet, that can give access to many of the databases worldwide[61]. UNIDO has also proposed setting up an International Biosafety Information Network, which would aim to have a contact in each member country. This contact would normally be a member of the authority controlling biosafety. In this way an efficient network of information will be established at the pivotal level of those involved in formulation and administration of biosafety regulation. Both information and expertise could be both formally and, perhaps even more important, informally exchanged though these channels. Lists of contacts in regulatory authority as well as those in companies intending to carry out releases will lead rapidly to dissemination of information, the rationalization of procedures and the elimination of duplicated effort. The emphasis of biosafety regulation must be in maintaining a safe posture founded on sound biological principles, and to use the information we have and will gain in the coming years around the world to refine regulation though the removal of unnecessary legislative burdens that address non-existent or unimportant perceived risks.

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GLOSSARY

Embryonic (ES) stem cell An undifferentiated cell derived from an early mammalian embryo that is able, after culture *in vitro*, to contribute to the tissues of a developing, recipient embryo. Such cells can be used for gene transfer.

Genetic manipulation Broadly, the use of recombinant DNA (rDNA) techniques to alter the sequences of DNA molecules. Two unrelated DNA molecules may be joined together to produce a molecule with novel properties.

Genome The complete DNA complement of an organism comprised of the sequences of all its DNA.

Genotype The genetic content of an organism, defined by its DNA sequences. The genotype determines many of the aspects of the **phenotype** of an organism. The genotype may be modified by classical breeding programmes or by **genetic manipulation**.

GMO Any organism modified by the enormous variety of techniques of modern molecular biology, from a cell of the gut bacterium *Escherichia coli* modified by bacteriophage transformation, through plants modified by a biolistic gun, to animals modified by **ES cell** incorporation.

Homozygous The state in which both copies of a gene (on the pair of chromosomes) are identical. Also may refer to pairs of chromosomes that are equivalent. If the two copies of the gene are dissimilar they are said to be **heterozygous**.

Microinjection Introduction of DNA (or, rarely, RNA) into the nucleus of a recipient cell. In animal transgenic biology, one of the pronuclei of the newly fertilized egg is microinjected with about 3 pl (3×10^{-9} ml) of DNA.

Mosaic An individual that contains cells of two or more genotypes. Such an individual results from introduction of **ES cells** into an embryo, or from integration of a **transgene** into only some of the cells of the very early embryo.

Phenotype The expressed characteristics of an organism, determined by the interaction between its **genotype** and the environment. Thus an organism that expresses growth hormone and is expected to grow more rapidly may not do so without an adequate supply of food.

Recombinant DNA (rDNA) DNA that has been modified using **genetic manipulation** to generate new characteristics.

Transfection (transformation) The process of altering the genetic makeup of a call by introducing foreign DNA. Typically, transformation is used to describe such introductions into bacterial cells, and transfection for introduction into animal cells. *In vitro* experiments use simple methods to transfer DNA into such cultured cells.

Transgene The DNA introduced into the **genome** of a recipient organism: typically used when that DNA is stably integrated into the host genome.

Vaccine Classically, an attenuated form of a disease-causing organism that confers immunity against infection by the parent, virulent organism. Recombinant vaccines typically cause production of the crucial immunity-inducing protein components in a non-pathogenic vector, most commonly vaccinia virus, the organism used as a vaccine for smallpox.

Vector A self-replicating carrier DNA (occasionally RNA) molecule into which foreign DNA can be inserted to allow its propagation and amplification.

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Table 1 A selected list of transgenic animals

Species	Transgene	Desired/anticipated phenotype	Comments	Refs
Mouse	MT/hGH	Increased growth	First dramatic demonstration of transgenic phenotype. Fertility impaired	12
Mouse	MT/GHRH	Increased growth	Fertility improved. More physiological?	13
Pig	MT/GH	Increased growth	Increased growth rate. Severe pathology similar to that found with injection of GH protein	14-16
Sheep	BLG/α_1-AT	Production of pharmaceutical in milk	35 to 60 g/l of protein	6
Goat	WAP/LAtPA	Production of pharmaceutical in milk	3 mg/l of protein. Purified 8000-fold	7
Chicken	RSV-MT/bGH	Increased growth	100 μg/l of protein	17
Chicken	ALV *env*	Viral resistance	Retrovirus-mediated RSV resistance	9

Abbreviations used: MT, metallothionein; hGH, human growth hormone; GHRH, growth hormone releasing hormone; BLG, β-lactoglobulin; α_1-AT, α_1-antitrypsin; WAP, whey acidic protein; LAtPA, long-acting tissue plasminogen activator; RSV, Rous sarcome virus; bGH, bovine growth hormone; ALV, avian leukosis virus; *env*, gene encoding the envelope glycoprotein.

Table 2 Routes to transgenesis in animal species

Method	Outline	Advantages	Disadvantages	Refs
1. Microinjection	DNA is microinjected with a pronucleus (mammals) or into the region of the nuclei (fish)	Established technique. Low frequency of mosaics. Expression well established. Usually stable integration	Complex, expensive equipment	2,5-7,25
2. ES cells	Embryonic cells established *in vitro*, are transfected with a DNA construct, analysed and introduced into a recipient embryo	Established technique (in rodents : livestock still experimental). Reliable, stable integration. Expression well established. Subtle modifications or gene ablation possible	Very complex. Fastidious cells. All transgenics of first generation are chimeric. Very expensive	22
3. Viral vector	An engineered vector is made into packaged virus using a helper cell line. Resulting defective virus particles used to infect host embryo cells	Established technique. Major technique if nuclear injection or ES cells not possible	Expression can be unstable. Concern about recombination with endogenous viruses. Concern about contamination with helper virus	9,17,23, 24
4. Sperm-mediated transfer	DNA is mixed with sperm, to which it binds. DNA transferred to eggs during IVF	Remarkably simple, no specialist equipment or culture	Unverified. Unstable integration? Unstable expression?	25-27
5. Electroporation	Cells in DNA are expressed to transient, high-voltage pulse, during which DNA enters the cell	Can be used for a variety of cell types. Reliable for cultured cells	Unverified in most animal cells, though some unpublished reports of success	

Table 3 Production of recombinant proteins

Host organism	Advantages	Disadvantages	Examples	Refs
Escherichia coli	Well-characterized, easy manipulation Good expression systems Simple culture	Protein usually remains in cells Protein may be aggregated or degraded Little modification	Insulin Interferon Growth hormone	35,36,37
Yeast	Long history of use Simple culture Good expression systems	Protein modification may be not accurate Protein may aggregate	Hepatitis antigen	38
Cultured mammalian cells	Export of modified proteins Good expression systems	Risk of contamination of culture Expensive culture	Erythropoietin Tissue plasminogen activator	39,40
Animals	Simple 'culture': self-replicating Accurate modification Some good expression systems	Difficult manipulation Little experience	α_1-antitrypsin Tissue palsminogen activator	5,6,7

Table 4 Potential hazards of release of GMOs

1. The Organism
   - The nature of the host
   - The stability and nature of the genetic modification
   - Laboratory testing and verification of the organism
2. The environment
   - The size location of site including ownership and security
   - Proximity to humans and other animals
   - The ecosystem of the release site and predicted effects
   - Release of any target biota (e.g predators), the known effects of the non-manipulated organism and xx effects of manipulated organisms
   - Numbers released at the site, the frequency of release and duration of release
   - Effects of the manipulation in behaviour of the organism in its natural habitat
   - Monitoring - how are the animals traced and for how long
3. Survival and spread
   - Susceptibility to artificial stress
   - Any details of modification designed to affect its ability to survive and to transfer genetic material
   - Potential for transfer of inserted DNA to other organisms and methods for monitoring that transfer
   - Elimination of superfluous organisms or DNA
4. Safety
   - Safety of the workers on site and their education
   - Contingency plans for unexpected effects of the transgenic organism
   - Physical containment and contingency plans in the event that this containment may be breached (for example, flooding of fish ponds)
   - Procedures for the termination of the experiment and disposal of manipulated organisms

Based on the recommendations of GENHAZ[51].

Table 5 A summary of GENHAZ

Components of the genetically modified system

Construct	-	the components of the rDNA
Recipient	-	the host organism
Product	-	the GMO

Stages of the release

MAKE or SELECT the recipient, prepare the construct and generate the product

RELEASE of the product into the environment

ESTABLISH: the period during which the product either establishes itself in the release environment, or fails to do so

POPULATION the pattern of growth, spread and reproduction that follows the initial period of establishment; the interaction of the product and the release environment

GENETIC TRANSFER: the unintended transfer of DNA

MONITOR: the monitoring of the release

TERMINATE AND CLEAN UP: plans for when the trial has been completed or if it must be terminated early

GENHAZ procedure

Apply guide words to generate DEVIATIONS

Develop CONSEQUENCES of each DEVIATION

Examine each CONSEQUENCE
Decide whether it requires ACTION and to avoid it
Decide whether it has a realistic cause

Decide what ACTION to take

Guide words

NO or NOT	a complete negation of the intention (eg a gene fails to insert into a vector)
MORE	a quantitative increase (eg the level of expression of a gene is greater than had been expected); could also be applied to time in terms of duration or frequency

LESS	a quantitative decrease (eg intended sterility of a transgenic animal is incomplete); could also be applied to time in terms of duration or frequency
AS WELL AS	a qualitative increase: something additional to the design intention happens (eg insects other than those targeted by a gene product are killed)
PART OF	a qualitative decrease: something less than the design intention happens (eg one of the genes inserted into the recipient fails to express)
OTHER THAN	something quite different from the design intention happens (eg the wrong construct is inserted)
WHERE ELSE	an intended event takes place in a location other than that planned (eg genetic material or the product of its expression occurs elsewhere than was planned)
WHEN ELSE	some effect appears at a time different from the expected (eg a modified animal reaches sexual maturity earlier or later than its unmodified form even though this was not the purpose of modification)

Based on the recommendations contained in GENHAZ[33].

Table 6 Checklist for legislation on release

1. What controls already exist?

   - novel exotic organisms
   - new strains of animals produced for food
   - new food or drug products

2. What is legislation designed to protect?

   - the consumer
   - the worker
   - the GMO
   - species that interact with the GMO
   - the environment

3. How novel are the products of genetic manipulation?

   - growth and reproductive regulation
   - disease resistance
   - increased stress tolerance, feed efficiency
   - production of novel proteins

4. What risks do such products imply?

   - genomic risks common to all breeding programmes e.g. more aggressive behaviour, wider ecological range
   - special risks arising from the nature of the rDNA e.g. stability, gene transfer, novel product, novel expression patterns

5. New legislation for transgenic animals

   - include all releases of novel or unfamiliar organism, not only GMOs
   - establish natural body of experts independent of proposers
   - use international experience (databases, previous releases) and expertise
   - legislate to be flexible, to simplify and generalize wherever possible on the basis of experience
   - engage public (the consumer) in the debate
   - respecting commercial confidentiality, keep assessment open
   - establish monitoring and termination protocols