Research report for DN
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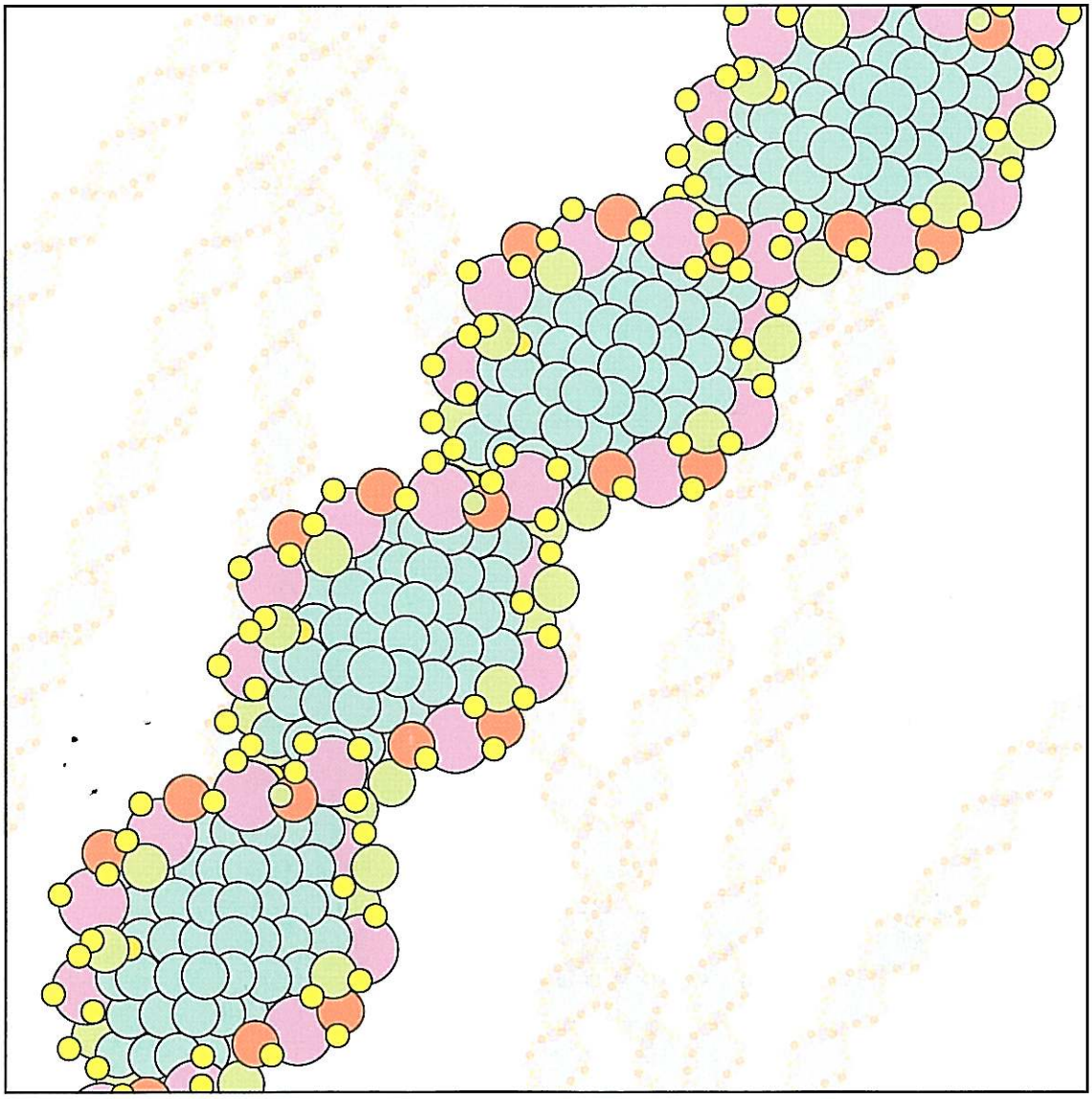
Too early may be too late

Ecological risks associated with the use of naked DNA as a biological tool for research, production and therapy



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Research report for DN 1999 – 1

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No. 1999 - 1

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Too early may be too late. Ecological risks associated with the use of naked DNA as a tool for research, production and therapy.
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Abstract:

This research report deals with naked DNA, and describes natural processes for gene transfer. Research and application of nucleic acids and naked DNA in gene technology, production, gene therapy and vaccination are also described. The research report discusses risks in connection with lack of knowledge and the present research on gene technology. Mechanisms for horizontal gene transfer within and between species, dispersal of naked DNA and RNA between virus, bacteria and higher organisms are discussed. The role of nucleic acids in an ecological and evolutionary connection is also discussed. The concluding chapter gives recommendations regarding necessary research effort on the background of the lack of knowledge within the field.

Ekstrakt:

Utredningen omhandler nakent DNA, og beskriver bl.a. naturlige prosesser for genoverføring. Samtidig beskrives forskning og anvendelsesområder for nukleinsyrer og nakent DNA innen genteknologi, produksjon, genterapi og vaksinasjon. Utredningen drøfter også risiko i forhold til mangel på kunnskap og den genteknologiske forskningen som foregår. Mekanismer for horisontal genoverføring innen og på tvers av artsgrenser, spredning av nakent DNA og RNA mellom virus, bakterier og høyere organismer diskuteres. Også nukleinsyrenes rolle i en økologisk og evolusjonær sammenheng drøftes. Det avsluttende kapitlet gir anbefalinger i forhold til nødvendig forskningsinnsats på bakgrunn av kunnskapsmangel innen fagområdet.

There is said to be a town somewhere where all the inhabitants are blind. An army commander once conquered it. He had a huge war elephant with him. None of the blind people had ever heard of such an animal and were very curious to find out what kind of creature it was. One night, they sent out their "scouts". Stealthily, they crept up to the elephant and formed a ring around it and tried to touch it. When they returned to the town, each one of them thought that he now knew something about the animal - because he had touched it and run his fingers across it. "Now?" their townsmen asked. "What kind of shape and appearance does an elephant have?" The first to answer was he who had touched the elephant's ear. He said: "It's a big, rough creature, broad, and shaped like a huge carpet." "Not at all," said he who had felt one of the feet. "In shape and appearance it's more like a pillar." But the man who had touched the elephant's trunk said: "You're both wrong. Because I've touched the animal with my own hands, and I know without any doubt what an elephant can best be compared with. It's like a huge chimney - a hollow pipe dangling freely in the air, without feet, without wings - and everyone can imagine what will happen if it's allowed to fly over our walls and breathe death and destruction around it…". Thus spoke these three blind men. Each one of them had touched a bit ….. one of many bits. But they had not managed to capture the whole.

Tor Åge Bringsværd: Gobi. Djengis Khan.
Gyldendal Norsk Forlag A/S 1987. ISBN 82-05-18668-5.

Foreword

The background for this project is the Directorate's management tasks in connection with modern biotechnology and responsibility for the natural environment. The Gene Technology Act regulates, among other things, the release of genetically modified organisms (GMOs) into the environment. When an application is made to release a GMO, an environmental impact assessment report has to be prepared dealing with possible health hazards and risks to the environment. In that context, there are many unanswered questions about the "fate" of the organisms and the genes that are inserted in them in the environment. Questions about the horizontal transfer of genes astride species boundaries and of uptake of naked DNA from the environment are at the forefront here. Naked DNA in the form of both round plasmids and strands have proved capable of "surviving" and remaining functional longer than was assumed previously. As the report shows, such genetic material can be integrated and expressed in higher organisms by injection. Knowledge of what happens to the genetic material in the environment when, for instance, micro-organisms break down biological material is largely non-existent.

This report was initiated on account of the lack of knowledge regarding ecological questions relating to naked DNA, nucleic acid molecules and horizontal gene transfer in the environment. The aim was to throw more light on the problem and offer recommendations to the environmental management authorities about the future need for research. Most molecular biological and gene technological research takes place in agriculture, industry and the biomedical sphere, and almost none in relation to environmental research. We believe that this report also covers problems and contains information that will be highly relevant for researchers and others working on gene technology methods and naked DNA in all these spheres of research.

The entire project has been in the hands of Professor Terje Traavik at the Department of Virology in the Institute of Medical Biology at the University of Tromsø, Norway, on behalf of the Directorate for Nature Research.

The Directorate now welcomes a revised version in English.

Trondheim, January 1999

Yngve Svarte
Director of the Department for Species Management

Preface

".....there's no doubt that any man with complete conviction, particularly who's an expert, is bound to shake anybody who's got an open mind. That's the advantage of having a closed mind".

John F. Kennedy.
Quoted from Richard Reeves: President Kennedy. Profile in Power.
Touchstone, New York 1993. ISBN 0-671-89289-4.

In connection with a research project concerning auto-immunity triggered by virus infection, we intravenously injected rabbits with naked viral DNA. Based on what was conventional knowledge about the fate of nucleic acids in the animal organism, we were relatively certain that such an injection would have no biological effect; the experiment was, in other words, meant to be a negative verification. We were therefore greatly amazed to discover that a full virus infection began among the rabbits. The injected DNA had actually reached the cells of the rabbits in an undamaged state, where it had been taken up and had its genes expressed.

This experience set in motion a mental process within me. In our laboratory, we were working with viruses, and we were working with DNA. From virology's infancy, we have had efficient laboratory procedures which protect both ourselves and the surroundings from being exposed to viral infections. In many of our experiments, we observed the powerful biological effects of naked DNA. Now it struck me as being paradoxical that DNA, in contrast to virus, is in some contexts handled and looked upon as if it were dead, biologically inactive molecules, even though our laboratory followed internationally recognised procedures for working with recombinant DNA.

Initially, I felt somewhat ashamed of my own lack of insight and overall knowledge. It was therefore personally consoling, but generally disturbing, to experience that all my colleagues to whom I spoke were completely incapable of understanding the problem until I explained the background and the whole context. Then I achieved meaningful discussion, heated reasoning and/or encouragement to undertake the task that now lies in front of you. In retrospect, this story illustrates yet again that the obvious is seldom seen in time, especially by "experts" like myself.

Because I know I am putting my head on the chopping block, I wish to emphasise as strongly as I can that I am in favour of gene technology and of research, manufacturing and resource exploitation based on molecular biology. I live on and for all this, and would therefore consider it tragic if accidents that could have been foreseen and avoided should slow down or even stop further development.

Nevertheless, at the same time, I am a father, a grandfather, a citizen and one who is help-ing to manage the planet we are living on. I believe that humanity can harvest long-term benefits from the new scientific and technological offers without exposing the frail ark, Tellus, and the life-forms it houses, to further trials. I also believe that our lack of know-ledge about both the ecosystems and the fate of nucleic acids within them must fortify our "act-on-the-safe-side" attitude and trigger new research which provides essential informa-tion. This is what this report is all about.

I wish to thank the Directorate for Nature Research, and Jan Husby in particular, for making this work possible, and for displaying patience when it became much more voluminous and, hence, time consuming, than was foreseen.

I am also indebted to my colleagues, Ørjan Olsvik and Steinar Johansen, for critically reading and constructively commenting on the text.

This work was carried out in addition to my ordinary obligations, during what should have been sorely needed leisure time. Because my family believed that the task was important, they encouraged me to carry it out and accepted that the little time we have together would be still more reduced. I therefore dedicate this work to them.

Regarding the new revised version in English much has happened in the fields of gene technology and genetically modified organisms (GMOs) since the first edition of this book was concluded in 1995. It is with a certain amount of regret that I have to state that my strong concerns have not diminished. These concerns are based on the "act-on-the-safe-side" attitude, or "precautionary principle" (see section 1.4), combined with an explicit appreciation of the general lack of knowledge among scientists with regard to the fate of recombinant nucleic acid constructs in the environment, and the complexity of the ecosystems into which they are released, or escape, for thereafter to function within.

The revision mainly consists in some new sections and the up-dating of relevant literature citations. It has not been necessary to revise or exclude most of the pertinent questions raised in the original, Norwegian version. Hence, my main conclusions, more research is needed, the precautionary principle should be practised, and slower commercialisation is called for, have not been shaken.

I am indebted to a number of persons who, more or less consciously, made contributions to this project. DN, represented by Jan Husby, initiated the process, and, once more, patiently awaited a product that emerged more tardily than planned. My friend and colleague Dr. Mae Wan-Ho has given strong support and encouragement, as well as engaging in fruitful conversations and discussions. My Ph.D. students, Anne Myhr and Hilde Hansen, have furnished literature, challenged and inspired me, and the "core group" of GENØK (Norwegian Institute of Gene Ecology), Drs. Dag Coucheron, Steinar Johansen, Ørjan Olsvik and Rolf Seljelid, has been a constant source of support and creative thinking.

I gratefully acknowledge Richard Binns, who performed the translation from Norwegian in an expert way, but had to wait a long while for the final product.

Tromsø, January 1999

Terje Traavik

Contents

Summary

The Introduction presents the perspectives on which this report is based; absence of evid-ence is never evidence of absence. It is realistic to assume that various recombinant and modified nucleic acid molecules will find their way into the environment in the years ahead. The question will then arise whether this will have any short-term or long-term negative impacts for the ecology, and whether, if so, it is possible for us to prevent or reverse any of these. The status of development and commercialisation of genetically modified organisms (GMOs) is also briefly outlined. Some examples of harmful effects that have already been seen, are discussed. The report gives no ultimate answers but, on the contrary, raises many new, and hopefully important, questions.

Chapter 2, entitled Man, gene technology and ecology, points out that we have utilised biological processes and changed genetic properties in domestic animals and useful plants through deliberate breeding over thousands of years. However, not before our own century have we been able to perform these and other kinds of genetic operations directly without having to rely on natural reproductive processes.

Gene technology may be viewed as an arsenal of biological, genetic, biochemical, chemical and physical techniques which simplify and make commonplace the manipulation and use of genetic material. Fragments of DNA or RNA can be isolated, cloned and amplified to attain quantities which facilitate studies of structure, function and expression, perhaps also changes which result in more desirable gene products.

We have already seen some of the advantages of gene technology. Products and proced-ures have been developed that are purer and less energy demanding, and fundamental scientific breakthroughs are continually being made that promise important advances in areas where they can be applied.

However, recent history provides some deplorable examples of our lack of ability, often also willingness, to make efforts aimed at predicting possible negative impacts of new technology on the environment and accommodating the technology to ecological reality. One problem in this context is that profits are achieved and utilised in the short term, whereas serious, damaging effects to the ecology develop slowly and may only be observed after decades or centuries. Maybe the use of gene technology will stand as a precedent-establishing example that mankind's sense of responsibility for life in the future is greater than his urge for short-term gain?

Chapter 3 briefly summarises the methodological basis for gene technology, the types of recombinant DNA molecules that are most commonly used (plasmids, bacteriophage DNA, cosmids, YACs, MACs), and what they are used for. Such molecules are often constructed so that they can replicate and produce proteins in both bacterial and mammal-ian cells. This has obvious implications should they escape. DNA from dead organisms or cells can survive in Nature and be taken up in new cells. The distinction between "naked DNA" and DNA from genetically modified organisms is therefore illusory in an ecological context.

Strategies for gene transfer (Chapter 5) summarises and evaluates how, through experi-ment, it is possible to ensure that nucleic acids (RNA or DNA) are taken up by living cells. Until recently, the most important transfer vectors were genetically modified viruses which can find their own way to appropriate host cells and transfer their genes to them. Several viral alternatives are available, all of which have their advantages and disadvantages. Because virus vectors always express some of their own genes in addition to the inserted gene, the possibility exists for non-desirable effects on the host cells. Naked nucleic acid vectors are consequently undergoing continuous development.

A number of chemical and physical methods have been developed to insert naked nucleic acid molecules into chosen target cells in cultures or living mammals. These range from micro-injection or the shooting of small projectiles directly into the cells (biolistics), via treatment with high-voltage electricity or heavy metal salts, to transport in tiny lipid capsules (liposomes) which can be constructed in such a way that the nucleic acids are only taken up by specific types of cell in an organism. For several of these methods, it is easy to envisage that similar conditions which can lead to cells taking up nucleic acids may arise naturally or as a consequence of pollution in the ecosystems. None of these methods can be performed in such a way that release of nucleic acids can be precluded. A great deal of research is being aimed at enhancing the uptake of nucleic acids by cells and the resistance to breakdown in the mammalian organism, thus making them more dubious in an ecological context.

Chapter 6 describes the revolution taking place regarding the use of naked, recombinant DNA, and also RNA, in multicellular organisms, animals and human beings. The discovery that plasmids injected into the muscles of mice resulted in expression of the genes which the plasmids carried was made as recently as 1990. It has subsequently been shown that the same can be achieved for other types of animals, including fish. Liposome-mediated transfer to animals under experimental conditions has achieved genetic expression in several organ systems following intravenous injection of plasmids. In some cases, this applies to the ovaries, which is a cause for deep concern because it includes the prospect of introducing nucleic acids that can be inherited. If such effects arise in animals or human beings through deliberate or unintentional release of nucleic acids, the results may be catastrophic.

A very important, broad-ranging area of application for naked nucleic acids is vaccination against infectious diseases. The introduction of, and genetic expression from, recombinant nucleic acids containing genes from disease-producing bacteria and viruses may give protective immunitive reactions on the same level as natural infections in human beings and animals, at the same time as some of the side effects accompanying the use of traditional vaccines are avoided. The DNA vaccines also have practical advantages that make them specially well suited for the poorest parts of the world. Development in this field has proceeded very rapidly. The first report of DNA vaccination of mice was published in 1993, and already in April 1995 a three-day international congress dealing exclusively with this subject was held in the USA.

The frightening development of antibiotic-resistant micro-organisms, and the disappoint-ingly slow development of antiviral chemotherapeutics, have led to new awareness of, and research related to, the application of vaccination as a treatment for infectious diseases. Promising results have been achieved. If these are reproducible and can be transferred to human beings and domestic animals,

they will lead to large quantities of naked DNA being produced and used in human and veterinary medicine.

Gene therapy implies that new genetic material (DNA or RNA) is transferred to the cells of an individual, providing a consequent therapeutic benefit for the individual. The object-ive will usually be to introduce one or more genes which result in the production of protein(s) of which the individual manufactures wrong versions or amounts, but it can also ensure that the production of damaging genetic products is prevented. Since every disease in man and animals, without exception, can be understood as changes in genetic expres-sion, it is not an audacious claim that gene therapy will revolutionise human and veterinary medicine. In principle, therapeutic gene technology can be used to change any kind of gene in any kind of organism. In the USA, several tens of gene-therapy protocols have already been approved for use on people, and more are being approved all the time. For the moment, an even wider distribution and routine application of gene therapy has been prevented by technical difficulties associated with achieving effective transfer of nucleic acids to individual organs and types of cell, maintenance of the required levels of genetic expression for sufficiently long periods and the danger of undesirable side effects as a result of the therapeutic nucleic acids being integrated into the host cell chromosomes. It may become possible to solve these problems by developing mammalian artificial chromo-somes (MACs). However, our understanding of key functions in the chromosomes seems too incomplete at present to permit the practical use of MACs. The application of con-structions based on the current level of knowledge will represent potential risks for health and the environment.

Inactivation or destruction of pre-determined nucleic acids without affecting the normal functions of the host cells would be an ideal strategy for treating, for instance infections and cancer in animals and humans. Such strategies may be developed on the basis of antisense- or ribozyme-techniques. In both cases, nucleic acids are used which are comple-mentary to, and can bind themselves specifically to, RNA molecules which are transcribed from specific target genes. The binding leads to the RNA molecules being inactivated or destroyed, and the protein for which the gene codes will consequently not be produced. However, if antisense or ribozyme molecules show unintended binding to host cell RNA in the target or some other kind of organism(s), unpredicted and terrible consequences may arise. Another problem with antisense molecules has been their rapid breakdown. Intensive research is therefore being carried out to modify them to achieve greater stabil-ity, but this will also make them more dubious in an ecological context.

Chapter 7 discusses a key issue in relation to ecological risks, the persistence of nucleic acids in Nature. Important questions in this context are the length of nucleic acid frag-ments that can persist for how long and under what conditions. Relatively little research has been carried out in this field. However, recent work has shown that chromosomal DNA from animals, humans and micro-organisms can survive in quite long fragments for thousands of years. Plasmids are also able to survive longer than previously assumed, but the actual survival time varies very substantially according to the environmental conditions to which the plasmid is exposed. There is, moreover, a great variation between different types of DNA under identical conditions. DNA that is adsorbed to particles is much more resistant to enzymatic breakdown than DNA dissolved in a liquid phase. Under natural conditions, long DNA fragments as well as plasmids may be detected for much longer periods than the micro-organisms themselves. Naked RNA has also proved to have a much longer survival time in Nature than previously assumed.

Mammalian cells can take up foreign nucleic acids in ways that are compatible with bio-logical activity, and this is the basis for transfections in cell cultures, gene therapy and DNA vaccination. When these procedures are used, foreign nucleic acids reach target cells along routes that avoid the natural boundaries which the macro-organisms have towards their surroundings. However, do, for instance under natural conditions, the epithelial sur-faces of animal gastrointestinal and respiratory tracts act as impervious barriers to the up-take of foreign DNA? And are there differences in this respect between types of nucleic acids and various environmental parameters, such as the actual types of pollution? Intuitive answers will be reassuring and comforting: macro-organisms are continuously in contact with large quantities of foreign DNA, and since this has gone on for millions of years it is quite inconceivable that, for instance naked recombinant DNA, can to a significant degree be taken up in biologically active ways and thereafter have any ecological or evolutionary effect.

Sections concerned with uptake of nucleic acids by the mammalian organism, and nucleic acid receptors, summarise the relatively limited knowledge available in this field. Some documented examples are known of uptake, and liberation, of foreign DNA in ways which may have great biological and evolutionary consequences. In other experiments, the added DNA was completely broken down. Hence, the problem is that we do not know the differ-ences between those DNA molecules and environmental conditions which form the basis for biological activity and those which do not. Most studies to date concern mammals; few data are available for other important groups of animals.

Major changes in the genetic material of individuals within species and populations take place regularly over time; DNA sequences can be copied, deleted, cut out and inserted into new positions, etc. Such changes are important for evolution, but may also lead to the death of embryos, develop-mental defects, hereditary diseases and cancer. These changes, and the mechanisms behind them, are summarised in Chapter 8. Until recently, it was thought that dramatic changes in DNA were usually caused by mating between complementary sequences (legitimate recombination). It has now been shown that illegitimate recombination, i.e. that which takes place between non-related sequences, is much more common than assumed. The mechanisms for initiating illegitimate recombination are large-ly unknown, and we will not for a long time, if ever, be able to control or prevent it. This phenomenon contributes to the feeling of uncertainty regarding the fate of naked DNA in the ecosys-tems.

The phenomenon of horizontal transfer of nucleic acids and genetic information is discuss-ed in Chapter 9. Horizontal (or lateral) gene transfer is defined as non-sexual transfer of genetic informa-tion between genomes, and consequently differs essentially from the verti-cal transfer from parent to offspring. DNA that is deliberately released or which escapes accidentally may theoretically be taken up by one type of cell in an ecosystem, and there-after be spread by horizontal transfer to other types of organisms and thence to new ecosystems. During the process, DNA may be integrated into the genetic material of separate individuals and thereafter be spread by vertical transfer.

Documented cases exist of genomic sequences that have been horizontally transferred from pro-caryotes to eucaryotes, from eucaryotes to procaryotes, between procaryotes and between eucaryo-tes. Horizontal transfer of genes is thus an undeniable fact, and one of the most important questions

that remains is whether such transfer can take place with a frequency and speed that affect evolution to any significant degree.

We know there are restrictions to the type of DNA that is capable of being transferred, but we do not know what kinds of mechanisms and sequences that characterise the DNA that is transferred. Consequently, we have no possibility of evaluating in advance whether a specific nucleic acid molecule will be horizontally transferred, when it will be transferred, or where it will end up.

It is obvious that evolution favours organisms that have a balance between genetic variation and stability, but we do not know how such a balance is established and maintained. Consequently, we also do not know whether and how the balance can be upset. Examples have been published which show that small changes in a DNA sequence can change the host spectrum of a transferable genetic element, and in this context it is important to remember that the recombinant nucleic acids we work with are usually of the type called shuttle vectors, i.e. they can be reproduced in both procaryotes and eucaryotes and be spread with both types of host organisms and across normal taxonomic boundaries.

There are great differences between cell types, and even between closely related organisms, with regard to how effectively a given nucleic acid sequence will be transferred. Differences in efficiency in excess of 25,000 times have been recorded between strains of the same bacteria. The transfer of different DNA molecules may vary in efficiency in the same organism, without any good explanation being available. It is also known that numerous naturally varying environmental factors, as well as pollution that varies in concentration, may affect the degree of horizontal gene transfer. It is easy to envisage naturally occurring vectors for foreign DNA such that a foreign genetic element passes like a relay baton from one vector type to the next, and thereby spreads across wide geographical distances and transgresses species' barriers. This has taken place with naturally occurring genes and parts of genes.

The very first transformation experiment, described in 1928, showed that dead, disease-producing bacteria could transfer their disease-producing genes to other bacteria following injection into mice. This illustrates the difference between phenotypical and genotypical death, and that functional DNA can have dramatic ecological consequences when intro-duced into new host cells.

Chapter 10 takes up the highly topical issue of whether pollution and other changes in the environment affect the transfer or detrimental effects of naked DNA. This is a question that has oddly enough been treated as a scientific orphan, which is alarming because, by analogy, it is easy to envisage that several types of environmental pollutants, so-called xenobiotics, may affect the properties of naked DNA directly in the form of higher muta-tion frequencies. This is valid for radioactive pollution, many herbicides and insecticides, as well as waste products from industrial and other forms of production. Mutations can be envisaged to be capable of changing the survival of nucleic acids and their ability to be taken up by, and expressed in, living cells. It is known that some of the same, and also other, environmental pollutants may change the surface membranes and metabolism of living cells in ways that may affect the ability and capacity of the cells to take up nucleic acids, and also the possibilities for nucleic acids to become biologically active, integrated into the cell's own DNA and to be carried on to its progeny or transferred to other types of cells within the ecosystem. This also applies to xenobiotics that act as, or counter, sex hormones. We know nothing about

how several forms of pollution interact, but we do know that the presence of several pollutants in the same environment is a reality today.

The concluding chapter makes it abundantly clear that when naked nucleic acids are used, too early may be too late because we know too little. There is a lack of knowledge inter-nationally, due to modest research efforts in areas associated with horizontal gene transfer. The background for this situation is understandable, but the situation is unacceptable.

It is also shown that exceptional efforts by pure and applied research groups throughout the world produce gene technology products and processes that are of great use. This is what these groups are good at; it is the positive opportunities of gene technology which stimulate enthusiasm, creativity and the will for effort. With a certain insight into how cre-ative processes and interactions take place, we know that the same research groups cannot make such exceptional efforts if they are compelled to carry out high-quality research on ecological risks and horizontal gene transfer at the same time as they develop procedures and products.

It is recommended that a national centre be set up to carry out research directed towards understand-ing the general mechanisms of horizontal gene transfer and enforcement of physical and biological barriers to the transfer of recombinant nucleic acids. This centre will thus carry out basic molecular biological and ecological research, and at the same time generate knowledge and methodology that can provide a basis for assessing risks in ad-vance. If it is to fulfil the tasks it is given, confidential and trustful forms of co-operation must be established with existing commercial and academic research groups. This should not create major problems because everybody involved has a primary goal in common: to utilise technology to the best advantage of humanity without compromising the spaceship Tellus.

1. Introduction: absence of evidence is never evidence of absence

Too early may be too late when the geni has been released, you've lost control of it and don't know how to get it back into the lamp.

Large amounts of recombinant or modified nucleic acids and oligonucleotides will be used for various tasks in the years to come. It is universally agreed that their escape is wholly undesirable. We can to some extent safeguard ourselves from escapes from laboratories and manufacturing facilities by physical barriers and good work routines, but accidents and technical faults that enable nucleic acids to escape will very probably occur. For some areas of use, like gene therapy and vaccination of animals and people, it will, in practice, be impossible to prevent nucleic acids from being dispersed from where they are used.

It may also be assumed that, as time goes by, released or escaped, genetically-modified organisms will leave behind them large quantities of naked, recombinant DNA when they die. We have become increasingly aware that nucleic acids can survive and retain biological activity long after the organisms have died.

All in all, it is thus realistic to assume that many different types of recombinant and modified nucleic acids may reach the environment. The question will then arise whether this can have any negative, short-term or long-term ecological impacts and, if so, whether we can prevent or remedy them.

This report is based on a systematic review, evaluation and interpretation of international literature concerned with the application of recombinant and modified nucleic acids, their ability to resist destruction, their uptake by cells and organisms, the biological consequences of such uptake, and the possibilities for and consequences of horizontal genetic dispersal within natural ecosystems.

The report gives no definitive answers; on the contrary, it raises many new, hopefully important, questions.

1.1 The present status of gene technology and genetically modified organisms (GMOs)

The potential benefits are well known, marketed by strong commercial interests and established scientific milieus. The hazards and risks are mostly hypothetical and may become evident in the long run. Hence, a number of pertinent questions related to basic insight, monitoring and risk assessment lack satisfactory answers (for reviews, see Ho, 1998; Ho *et al*., 1998, Nielsen *et al*., 1998). This, in itself, calls for the "precautionary principle" to be invoked and for greater research effort.

A large number of plant, and some animal, species have been genetically modified (GM) by recombinant DNA techniques, methods included in the collective term "gene technology". In the USA, about 40 genetically modified plant species have been approved for commercial use. After being introduced in 1996, 27% of soya beans are now herbicide-resistant, and about 25% of maize is assumed to be genetically modified (Williams, 1998). Herbicide tolerance (54%), insect resistance (37%) and virus resistance (14%) make up the vast majority of gene modifications, while quality improvements with regard to growth and nutrient composition represent less than 1% (James, 1997). This is because the trans-national manufacturing companies want to offer packages of their own pesticide and GM plants which tolerate it (Ho, 1998).

The production, marketing and consumption of GM food is highly controversial. The controversy mainly concerns whether the first generation GM organisms should be commercialised at all, and to what extent, and how GM food should be labelled. In the EU, serious conflicts of interest and opinion are seen within and between member countries. The United Kingdom, Austria, Luxembourg, France and Greece currently have various kinds of moratoria on GM plants, and the Environmental Advice Committee of the European Parliament has recently called for a limited moratorium. Organic plant breeders in various EU countries have taken governmental and commercial institutions to court, because scientifically based risk assessments cannot prevent cross-pollination from GM crops from taking place. In the USA, commercialisation of GM plants has met little opposition from the government or consumers (Williams, 1998), and in Scandinavia there is a virtual absence of public debate, in itself a risk factor.

Experience with large-scale production and consumption of GM organisms is, of course, very limited. Proponents of early GM plant commercialisation focus on the hypothetical advantages, especially in the context of the critical global nutritional situation (Brown, 1997), and the suppressive effect on chemical pollution in all forms of primary production. The opponents bring forward lack of knowledge, uncertainty and potential hazard to public health and the environment, and insist that the "precautionary principle" should be invoked (Rissler & Mellon, 1996; Ho, 1998). Both parties are fighting for the attention of politicians, "experts", dealers, consumers and public opinion.

1.2 Risk factors and hazards

To carry out a genetic modification, a recombinant, genetic vector has to be constructed (see Chapter 3). The vector is intended to carry the cloned gene safely into the chosen organism and order the gene to become expressed, i.e. produce the protein for which it codes.

A vector will, in addition to the chosen gene, be composed of a number of other DNA elements. Typically, it needs a control element (promotor or enhancer) to express the gene, and an extra gene coding for resistance to an antibiotic or some other cytotoxic substance. An alternative to the highly controversial antibiotic resistance genes for GM plants is a gene (csr-1) that provides resistance to the herbicide chlorsulfon (Bergelson *et al*., 1998).

Vector DNA molecules are transferred across cell membranes by the aid of biolistics ("gene cannons"), chemical treatment or exposing cells to an electric field (see Chapter 3). The vector mole-

cules are then transported to the cell nucleus to become inserted (integrated) into the chromosome(s) of the recipient cell. Integration takes place at unpredictable locations in the chromosomes.

Vector transfer techniques are inefficient. Only a fraction of the treated cells take up and express the chosen gene. An antibiotic is added to select the vector-containing cell population. Cells not expressing the vector resistance gene are then killed. Surviving cells are the basis for the development of GMOs, in which all the cells of the organism contain integrated vector DNA and express the required gene.

1.2.1 Genetic modification differs from traditional cultivation and breeding

It is often argued that genetic modification represents a more precise, but not fundamentally different, kind of breeding or cultivation. This must be rejected, because:

- Unnatural recombinations are created. Genetic material is recombined between species for which there is no, or very little, probability of natural progeny.

- New, exotic genes or DNA sequences are introduced into unpredictable chromosomal locations. Conventional breeding shuffles around aberrant versions (alleles) of the same genes, while these are fixed in the chromosomal locations they have been given by evolution. Gene technology introduces new, exotic genes whose location within the recipient cell DNA is unpredictable and cannot be targeted. This may result in unpredictable effects on the metabolism, physiology and biochemistry of the recipient, transgenic organism that are incapable of being detected with traditional methods of control.

- The vectors used are efficient genetic parasites. They are gene shuttles developed to move and express genes across species' boundaries and ecological barriers:
  i) They are mosaics of genetic elements and sequences derived from most efficient genetic parasites (viruses, plasmids, mobile elements). Many of them are able to invade and insert their DNA into the chromosomes of any kind of cell, with possible genetic or metabolic harm as a consequence.
  ii) They are specifically constructed to break species' barriers. In transit, they may pick up and transfer genes from new host organisms or their genetic parasites. Such newly created genetic mosaics may then become transferred to new species, or recombine between them to result in pathogenic viruses with potential to infect earlier refractory hosts, etc. During such relays, genetic rearrangements and mutations may arise at any given time, with unpredictable results.
  iii) They carry resistance genes which, in themselves, may represent new, or enhance existing, public health and environmental problems (i.e. antibiotic resistance in pathogenic bacteria or herbicide-tolerant "superweeds").

1.2.2 Changes in GMOs or their products

The most serious scientifically based arguments against large-scale, commercial use of first generation GMOs are based on the unpredictability of *where* vector DNA will be inserted in the recipient

cell chromosomes. The consequences of insertion may vary considerably depending on the precise insertion location (Doerfler *et al*., 1997). This is valid for the expression of the inserted transgene as well as for changes in the recipient organism's own genes and their expression levels.

Some of the most prominent uncertainties are related to the fact that the recipient organism has received a new promotor or enhancer. These elements govern the gene expression levels of their attached transgenes, but after insertion they may also change the gene expression and methylation patterns in the recipient chromosome(s) over long distances up- and downstream from the insertion site. Promotors and enhancers function in response to signals received from the internal or external environment of the organism. For a GMO this results in unpredictability with regard to:

- the expression level of the inserted foreign gene(s)
- the expression of a vast number of the organism´s own genes
- the influence of geographical, climatic, chemical (e.g. xenobiotic) and ecological changes in the environment
- the transfer of vector sequences within the chromosomes of the organism, and vertical and/or horizontal gene transfer to other organisms.

1.2.3 Changes in ecosystems and the environment in a broad sense

Genetic pollution from GMOs is a real option and can be caused by cross-pollination, unplanned breeding and horizontal gene transfer (see Chapter 8). Such events may result in extensive and unpredictable health, environmental and socioeconomic problems. Environmental persistence and transfer of nucleic acids is extensively discussed in later chapters. The issue has gained added reality after a highly respected research group demonstrated that, under some circumstances, ingested DNA may be taken up from the intestines of mice, inserted into chromosomes and vertically transmitted to offspring (Doerfler *et al*., 1997, 1998; Schubbert *et al*., 1997).

1.3 Risk and assessment of risk

The term "risk" is very often confused with "probability" and, hence, used erroneously. Risk is defined as the probability that a certain event will take place, multiplied by the consequences arising *if* it takes place. The atomic bomb makes a good basis for conceiving the content of the term. With regard to the development and commercialisation of GMOs, we are often unable to determine either the probability of unintended events taking place or their consequences. Hence, our present state of ignorance makes scientifically based risk assessments impossible. This calls for the "precautionary principle" to be invoked.

1.4 The precautionary principle (Norwegian: "Føre-var prinsippet")

This principle is now established in international declarations and agreements. It was introduced as an ethical road sign. It implies that responsibility for future generations and the environment must be combined with the anthropocentric needs of the present.

In the context of gene technology and the use of GMOs, a general definition might be:
"In order to obtain sustainable development, politics should be based on the precautionary principle. Environmental and health policies must be aimed at predicting, preventing and attacking the causes of environmental or health hazards. When there is reason to suspect threats of serious, irreversible damage, lack of scientific evidence should not be used as a basis for postponement of preventive measures" (revised after Cameron & Abouchar, 1991).

1.5 Documented hazards and risks

During the short period GMOs (mostly plants) have been employed, a number of warning signals have already emerged.

1.5.1 Changes in the GMO or its products

- For a long while, manufacturers of genetically engineered bovine growth hormone (BGH), injected into cows to increase milk production, claimed that it was identical to its natural counterpart. Independent research subsequently demonstrated that epsilon-N-acethyllysine was being substituted for lysine in the engineered hormone (Violand *et al*, 1994). Such amino acid substitutions may have unpredictable consequences for the conformation and functions of proteins. Recently, indications have been published that milk from cows treated with BGH may contribute to enhanced risk of mammary cancer by increasing the concentration of IGF-1 in milk (Outwater *et al*., 1997; Gebauer *et al*., 1998; Hawkinson *et al*., 1998).

- Tobacco plants were genetically engineered to produce gamma-linolenic acid. Instead, the plants mainly produced the toxic product octadecatetraenic acid. Unmodified tobacco plants do not contain this substance (Reddy & Thomas, 1996).

- When yeast was genetically modified to obtain increased fermentation, it was unexpectedly discovered that the metabolite methyl-glyoxal accumulated in toxic and mutagenic concentrations (Inose & Murata, 1995).

- When a gene from a brazil nut was inserted into soya bean plants, unexpected, strong allergic reactions were recorded in nut-allergic persons who had never had any problems with soya bean products. The inserted gene did not code for any known allergen (Nordlee *et al*., 1996).

- A bacterium (*Bacillus amyloliquefaciens*) was genetically engineered to produce increased levels of the amino acid L-tryptophan, which has widespread application in tablets used as a nutritional supplement. Small amounts of a toxic, tryptophan-related molecule were identified in the tablets (Sidransky *et al*., 1994). Whether this was the cause of EMS (easinophilia-myalgia syndrome), which resulted in 37 deaths and 1500 cases of chronic neurologic and autoimmune symptoms, has never been clarified, mainly because the genetically modified stock of bacteria was not available for investigation (Australian Gen-Ethics Network, 1994).

1.5.2 Environmental effects

- Researchers at the Scottish Crop Research Institute in Dundee have demonstrated indirect ecological effects from GM potato plants. The plants expressed an inserted lecthin gene to reduce aphid attacks. Ladybirds predating aphids containing lecthin had their life expectancy and reproducibility signifi-cantly reduced. Likewise, researchers at the Swiss Federal Research Station for Agroecology in Zürich have demonstrated serious harm to lacewings foraging on aphids affected by the insecticide Bt toxin produced by GM maize (Williams, 1998). It is already a major, worldwide agricultural problem that natural predators of crop-ruining insects are disappearing. An acceleration of this process would be tragic.

- Field trials in Denmark and Scotland have shown that GM oilseed rape may transfer its inserted transgene by cross-pollination of wild relatives (Mikkelsen *et al*., 1996), and experiments in France have demonstrated the transfer of resistance genes from rape to radish (Chévre *et al*., 1997). Similar examples, with the spread of transgenes over long distances, have been demonstrated for other GM plant species. Organic plant farmers in European countries have initiated legal actions for this reason. When their farms are situated close to fields with GM crops, their products may be deprived of the “organic” labelling.

- Recently, it was demonstrated that self-pollinating GM plants may have a forced, augmented capability to cross-pollinate other plants, resulting in the transfer of inserted transgenes (Bergelson *et al*., 1998). The unpredictabilty of this process was demon-strated by the fact that identical, inbred plants genetically modified in separate experi-ments had differing abilities to cross-pollinate other plants. Although the experiments were carried out on a single plant species, *Arabidopsis thaliana*, these results have general interest, partly because the inserted gene (*csr-1*) has been introduced into various plant species as an alternative selection marker to replace antibiotic resistance genes.

- GM cotton plants with inserted herbicide tolerance genes have shown two types of malfunction. In some cases the plants dropped their cotton bolls, in others the tolerance genes were not properly expressed so that the GM plants were killed by herbicide (Fox, 1997). The manufacturers blamed extreme climatic conditions, indirectly refuting claims of unpredictability put forward by opponents. A number of cotton farmers pressed charges. The manufacturers offered economic settlements out of court.

1.6 Do the procedures for GMO development deserve the label "technology"?

"Technology" is derived from the Greek term "tekhne" which is connected to handicraft or the arts. Our associations with the word include predictability, control and reproduci-bility. The parts of gene technology which concern the construction of vectors are truly technology. However, current techniques for genetic modification of cells and organisms mean:

- No possibility to target the vector or transgene to specific sites within the recipient genomes. In practical terms this means that modifications performed with identical recipients and vector gene constructs under the same standardised conditions may result in very different GMOs, depending on where the transgenes become inserted.

- No control with changes in gene expression patterns for the inserted or endogenous genes of the GMO.

- No control on whether the inserted transgene(s), or parts thereof, move within or from the recipient genome, or where transferred DNA sequences end up in the ecosystems.

2. Man, gene technology and ecology

"Humans will increasingly need to be perceived as products of their evolutionary past, healthy insofar as the niche they occupy remains as it recently was, and for a while, substantially is. The niche is vast, virtually the entire world, but we have outstripped the protection its size once gave us, and it."

Jennifer Leaning

The processes that have entirely transformed the Earth's ecosystems during the past 50 years have not been given any kind of heroic name in line with "The Industrial Revolution". Because of the environmental pollution and energy consumption caused by these processes, concepts such as "global climatic changes", "habitat destruction", "loss of biodiversity" and "irreversible tapping of natural resources" have become integrated into our language and have to be taken into consideration when we are evaluating the state of health of the Earth and its peoples (Haines, 1991; McMichael, 1993; Chivian *et al*., 1993; Leaning, 1994).

Man has attempted to make use of biological processes for thousands of years. The ability of yeast cells to store alcohol was exploited for beer brewing by the Sumerians and Babylonians more than 6000 years B.C. Man has been trying to manipulate the genetic constitution of animals and plants by cross-breeding for nearly as long. A Babylonian clay tablet from about 4000 years B.C. shows how characteristic traits in the head of the horse were maintained through five generations. Petroglyphs from ancient Egypt show men undertaking cross-pollination of date palms with the obvious intent of improving the quality of the fruit (Thakur *et al*., 1991).

Nevertheless, only during the present century has mankind learnt how to deliberately alter the genetic content of organisms, *directly* - without being dependent on reproductive processes. During the same period, we have fundamentally changed the "basal physiology of the planet" (Cortese, 1993).

X-rays helped to induce DNA mutations in the laboratory as early as 1927, and a vast repertoire of mutagenic radiation techniques and chemicals has subsequently been developed. In the 1960's and 1970's, a quantum leap was taken in the ability of man to manipulate the genetic content of cells, culminating in reports of the first successful genetic cloning experiments in 1972-73 (Jackson *et al*., 1972; Cohen *et al*., 1973; see the review by Antebi & Fishlock, 1986). This led in turn to the development of gene technology - or recombinant DNA technology - which includes methods and techniques with potential advantages and risks for mankind, the environment and Tellus, our common home. Gene technology can be looked upon as an arsenal of biological, genetic, biochemical, chemical and physical procedures which simplify and generalise the localisation, isolation, characterisation, modification, synthesis, transfer and expression of genetic material. In the course of two decades, this has revolutionised molecular biology, genetics, biotechnology and biomedicine. Today, virtually any DNA or RNA fragments can be isolated and multiplied to amounts which enable studies of structure, function and expression. Production units can be established based on living cell

systems which store large quantities of special biological substances. Recombinant DNA techniques have recently been applied in industrial tasks to produce economically valuable substances of high purity.

Some of the advantages of gene technology have already been seen in the form of new, often purer, less contaminating and less energy-consuming products and processes, especially within the fields of medicine and agriculture (both plants and animals). In addition, there are the fundamental scientific breakthroughs that have taken place within a number of biological disciplines and fields, with built-in promises of new advances in the application of the method.

2.1 Poor track record

However, man has a poor track record when it comes to predicting the effects of new technology on the environment and his ability to place technology in an ecological context (Thakur *et al*., 1991).

Technology is developed to achieve benefits and there are many tragic examples of how people, elated over these, have both overlooked and neglected to adequately investigate the possibilities for dramatic disadvantages, which have therefore first been acknowledged much later.

Frightening examples from the last half of the 20th century include the application of chlorinated hydrocarbons to combat plant pests (see Chapter 9), and the "peaceful" exploitation of nuclear power. We know now that the environment on the Earth has been seriously damaged by these senseless encroachments on the ecosystems, but it will still be a long time before we are able to recognise *how* serious the damage is.

In both these cases, sectors of informed public opinion in many countries serious questioned their safety and warned of potential side effects. The research communities on the other hand, with a few brave exceptions, naïvely and optimistically gave their development their full support, and were unanimous in their view that there were no real risks of undesirable effects for health and the environment. The same experts and research milieus that had participated in developing the new technology were commissioned as advisors by political authorities in connection with the pre-assessment of risks and the setting-up of systems to record damage. Researchers are people like everyone else. The ability for critical and objective evaluation of risks associated with a person's own lifework is not a predominant part of human nature. There was, and still is, a lack of competent, independent expertise in many technological fields.

Recent years have seen many examples of unforeseen side effects from "safe" technology and biological combat methods having led to health risks and threatened to disturb the ecological balance. The following examples of accidents and erroneous evaluations have in common that the consequences are still developing and we still do not know the full extent of the damage.

2.1.1 Antibiotic resistance genes

- The development of *bacteria that are resistant to antibiotics* now represents a brewing catastrophe. Multiresistant strains of bacteria containing genes that are resistant to, for example plasmids, do not remain in hospitals but have now spread to the "healthy" community and even to large numbers of free-living, naturally occurring species of bacteria (Davies 1994; Kruse, 1994: Kruse & Sørum, 1994; Thomson *et al*., 1994). Antibiotics have saved countless lives, prevented suffering and preserved food resources and valuable resources in animal husbandry and aquaculture. However, the senseless use of antibiotics has simultaneously resulted in microbes now being on the warpath. Strains of more and more species of microbes that are important for medicine and veterinary medicine are proving resistant to all relevant antibiotics. "Old" infectious diseases, such as tuberculosis, are returning, and bacteria living freely in the ecosystems have acquired resistance to antibiotics. During the last few decades, confidence in antibiotics has, moreover, led to the stagnation of research into and testing of alternative strategies for preventing and treating infectious diseases. These fields of research now have to be revitalised, because no-one, including the pharmaceutical industry, believes that the constant development of new antibiotics is the right path to take. Horizontal gene transfer of antibiotic genes lies at the root of the problem (see Chapter 8).

2.1.2 Recombinant plant viruses

- In the last decade, researchers have been eager to *make plants resistant to viral infections* by inserting virus genes in the plant genome. If, for example, the gene which codes for the coat protein for the cowpea chlorotic mottle virus (CCMV) is inserted in plants, the plants become resistant to both CCMV and several other related viruses. It has now been shown that when such transgenic plants are infected with other viruses new, recombinant viruses can arise which have had their host specificity and other biological properties changed (Greene & Allison, 1994). This possibility, and the need to investigate it, had been pointed out by critically inclined scientists for many years, but their protests had been drowned by representatives of both the biotechnological industry and the research community who were optimistically eager to develop the technique. Even after Greene and Allison's results were published, such experts attempted to undermine the significance of the discoveries without having alternative results of their own to point to (Falk & Bruening, 1994). Incidentally, the story did not end here. In further work, Greene and Allison demonstrated that a targeted trimming of the viral transgene seemed to eliminate the development of viral recombinants (Greene & Allison, 1996). This illustrates the importance of invoking the "precautionary principle" to gain time to identify risk-imposing mechanisms and look for means to prevent them.

- A striking example of an entirely unexpected effect was observed in connection with the *release of soya plants* grafted with genetically modified *Bradyrhizobium japonica* bacteria in Louisiana, USA. Small-scale greenhouse trials suggested that genetically modified variants were out-competed, but when outdoor release took place the experimenters were surprised to find that the opposite was true. Eager biotechnologists had claimed beforehand that outdoor trials were unnecessary, because the results of the greenhouse trials were so unambiguous (Cairns & Orvos, 1992). In that particular case, it was possible to look for an obvious phenotype, but that has not always been available in all the

outdoor trials carried out afterwards, which amounted to 2000 between 1992 and 1994 in the USA alone (McNally, 1994), and has now reached approximately 15,000 (Nielsen *et al*., 1998).

- The brush-tailed possum (*Trichosurus vulpecula*) was imported into New Zealand in 1837 as a basis for the fur industry. It has now become a serious pest, for two reasons. Firstly, possums are the most important reservoir for bovine tuberculosis, an economically important illness that is becoming ever-more widely dispersed in New Zealand. Secondly, the possum population has now multiplied to such an extent that it poses a threat to natural woodland trees. So far, attempts have been made to control the problem by poisoning the animals with sodium mono-fluoroacetate placed in bait. However, this in itself is ecologically unacceptable since the bait is eaten by a number of other species. It has therefore now been suggested, perfectly seriously, to fight the possum by releasing a herpes virus that is transmitted sexually and produces sterility in the infected females (Barlow, 1994). The host specificity of this virus has been poorly studied and documented. The possibility for recombination with other herpes viruses which circulate within the ecosystems to which the possum belongs has not been investigated at all. This is an admirable illustration of how *one* poorly thought-out encroachment on ecology may lead to chain reactions of measures with unforeseeable, but potentially undesirable, environmental impacts.

- In 1956, 54 African queen bees (*Apis mellifera scutellatus*) were imported to Brazil from South Africa and Tasmania to cross-breed them with the more sedate European bees. The intention was to produce honeybees that tolerate more heat. An accident caused 26 swarms of hybrid bees to escape from a laboratory in Sao Paulo. Since then, these "African killer bees" have spread northwards. The migration front reached Panama in 1982, Mexico in 1987, Texas in 1990 and California in 1993. So far, more than 1000 people have died after being stung by these Africanised bees (Gorman, 1994), about twice as many as the Ebola and Marburg virus infections together.

- The story of the rabbits that were released in Australia is old and well known. The rabbits lacked predators and multiplied into huge, destructive populations. Attempts to regulate the populations using the myxoma virus seemed initially successful. However, only apparently so, because resistant rabbit populations soon evolved, as also did virus strains that were less capable of developing disease.

2.1.3 Be aware of dogmas!

- Dogmas concerning absence of hazards have often been proven wrong (e.g. the Titanic). A relevant example is the belief that DNA in food and forage cannot be taken up from the gastrointestinal tract. This belief was supported by some experimental studies, and the whole evolutionary history as well as our daily intake of vast amounts of DNA from various sources goes to prove it. Absolute biological and ecological truths are, however, very rare, and rare phenomena may have important consequences when they take place. Recently, this was illustrated by the demonstration that following ingestion by mice, DNA from the M13 bacteriophage could be detected as relatively long fragments in fæces, peripheral leukocytes, spleen and liver cells in significant time intervals after feeding. In the cells, the ingested M13 DNA was found in a chromosome-integrated form (Doerfler *et al*., 1997; Schubbert *et al*., 1997). When such DNA was fed to pregnant mice, the test DNA was detected in various organs from foetuses and newborn animals (Doerfler & Schubbert, 1998). The experimental

conditions strongly indicated that the DNA had been transferred across the placenta. The authors conluded that the consequences of foreign-DNA uptake in the context of mutagenesis and oncogenesis should be subject to controlled experiments. Such experiments have still not taken place. Another unclarified issue is connected with the detection of long M13 DNA fragments in the faeces (Schubbert *et al*., 1997). If such fragments are taken up by enetric bacteria, unwanted establishment of sequences from transgenes, i.e. antibiotic resistance genes, may take place in pathogenic or opportunistic bacteria.

- Another striking example is represented by the BSE (bovine spongiform encephalopathia) story. Against the explicit conclusions of experts, the BSE prions crossed the hypothesised "species" barrier and initiated new variant Creutzfeld-Jacob disease (nv CJD) in human beings. Recently, it has been demonstrated that a vast number of BSE prion-carrying, symptom-free cattle may have been consumed, and at the moment the extent of nv CJD is impossible to forecast.

In these and many other cases, some of which are mentioned in Chapter 1, the experts were wrong. To the extent that any prior investigations of damaging effects had been undertaken, methods and approaches had been used that were only capable of disclosing short-term effects, whereas in ecological contexts it is long-term impacts that are most important and most serious (Leaning, 1994; Colborn *et al*., 1994). *Long-term impacts in these contexts, and also in connection with the possible damaging effects of dispersal of naked DNA, means scales, not of months or years, but of at least ten to hundreds of years.*

We are soon putting behind us the bloodiest century in the history of mankind. In our technological fervour, in our inquisitiveness and in our pleasure at being able to rub magic lamps, we have made enormous, and still unpredictable, erroneous assessments of our relationship to our surroundings and the, relatively speaking, ever smaller vessel in which we are all sailing.

Is the use of gene technology to remain as a historical turning point, the first example of mankind's feeling of responsibility for life in the future being stronger than the urge for short-term advantages? Can the precautionary principle be invoked in this area?

3. What do we mean by "naked DNA"?

3.1 To be naked or to become naked

Nucleic acids may escape from laboratories, manufacturing facilities or in connection with prevention or treatment of illnesses (see Chapter 5). Generally, such DNA is intended to be used in, or is a result of, gene technological manipulations. At the outset, such DNA *is* naked.

However, we now have an increasing need to recognise the difference between the situation where genetically modified or non-modified organisms no longer maintain their life functions (they are phenotypically dead) and where their DNA has lost its biological activity (is genetically dead). DNA can often be identified in a biologically active form long after the cells in which it once resided have died and been broken down (see the reviews by Lorenz & Wackernagel, 1994 and Nielsen *et al*., 1998). Such DNA *becomes* naked, but can otherwise be looked upon in the same way as the first group in an ecological context.

3.2 Gene technology

The basis for gene technology is the methods for cloning and multiplying special genes, or DNA sequences, and thereafter being able to get the genes expressed in the form of proteins within living bacterial, plant, insectean, or mammalian cells. The objective, for example, may be to produce the protein for application (e.g. insulin, or human growth hormone, as medicine), or to study the effects of the protein on a cell which does not usually produce it.

Both multiplication and expression are first achieved after the DNA sequence, or gene, has been removed from its usual position in, for instance, a human chromosome, and glued into a DNA carrier molecule, a *vector*. The process of removing a DNA fragment from its usual location and gluing it into a vector results in a *recombinant DNA* molecule. The controlled removal is undertaken with the help of *restriction enzymes (restriction endonucleases),* and the gluing is performed with DNA *ligaces*. When the recombinant vector has been placed in a host cell and has multiplied, the entire process that has been performed is termed a *molecular cloning*.

The original DNA fragment may also have derived through nucleic acid amplification methods like *PCR or LCR* (Kolstø & Prydz, 1994) and others (e.g. NASBA; Kievits *et al*., 1991). When these techniques are used, for instance for diagnostic purposes, they entail a huge *multiplication of naked DNA molecules which, in themselves, may result in ecological risks*. This is obvious when genes that are resistant to antibiotics, or microbial pathogenetic genes are copied in vast numbers, but it can also apply to many other amplification products.

Different objectives may require that identical DNA fragments, or genes, are passed from vector to vector like a relay baton, i.e. they are recloned, thus, for example, first achieving multiplication and then the desired degree of genetic expression in different types of host cells. Carrier DNA fragments, used exclusively to multiply desired fragments, are often called *cloning vectors*, whereas those used solely to get the cloned gene expressed as protein are termed *expression vectors*. Many of the vectors that are used can, in practice, be used for both purposes. Vectors which are able to multiply, and perhaps also provide genetic expression in both eucaryotic and procaryotic cells, are called *shuttle vectors*.

Irrespective of the purpose which the vectors are intended to serve, they themselves are recombinant molecules that are traditionally constructed on the basis of known forms of self-replicating DNA.

3.3 Vectors

A major problem with many types of vectors, and hence also GMOs, is that they contain genes that confer resistance to antibiotics, herbicides, insecticides and other cytotoxic products, and these can be spread in the environment and create ecological problems.

3.3.1 Plasmids

Bacterial plasmids are double-stranded, enclosed, circular DNA molecules which may vary in size from 1 kbp to several hundred kbp. They are found in a large number of bacterium species and behave as self-replicating units outside the bacterium chromosome. The plasmids are dependent upon enzymes and other proteins which are coded for and produced by the host cell, but they themselves may contain genes whose products are advantageous for the host bacterium under special circumstances. Plasmid-coded proteins can provide resistance to, or production of, antibiotics, degrade complex organic compounds, act as toxins, etc. When they are utilised as vectors, the plasmids contain selection markers, polycloning seats with recognition sequences for up to some twenty restriction enzymes, genes which provide opportunities for differentiating between molecules that have taken up the desired fragment and empty vectors, as well as sequences which provide multiplication and genetic expression in procaryotic and/or eucaryotic host cells. In principle, there are no limits to the size of DNA fragments that can be cloned in plasmids, but when the plasmid exceeds a total size of 15-20 kbp its efficiency for transformation, and hence usefulness, is often drastically reduced.

Plasmids used for DNA vaccination, insertion of genes into cells and organisms, and for gene therapy, are usually shuttle vectors. They contain DNA sequences which ensure replication and genetic expression in both procaryotic and eucaryotic cells. Hence, if they escape they may multiply within and become spread by representatives of both kingdoms.

3.3.2 Bacteriophage λ (lambda) vectors

Foreign DNA can be cloned into this genome of this bacterial virus and be introduced into host cells, after which the recombinant virus genome will multiply and be packed in virus particles, which, in turn, can be used to infect new host cells for further multiplication of the desired DNA fragment. Vectors based on bacteriophage λ are also found which not only permit multiplication of foreign sequences, but also allow genetic expression from these in bacterial cells. The best known of these vectors is λgtl l, but many other λgt vectors are known. DNA fragments up to about 24 kbp may be efficiently multiplied within bacteriophage λ vectors.

3.3.3 Cosmid vectors

In their simplest form, cosmids are modified plasmids which carry a copy of the DNA sequences (*cos* sequences) that are necessary to pack DNA into bacteriophage λ particles. Because the cosmids have a bacterial replication origin and an antibiotic resistance marker, they can be introduced into bacteria by standard transformation methods and be multiplied like plasmids; they can also be packed as bacteriophage particles. DNA fragments of between 33 and 47 kbp can be cloned in cosmids. A few cosmid vectors contain eucaryotic replication origins, and can therefore multiply autonomously if they are introduced into mammalian cells.

3.3.4 YAC (yeast artificial chromosome) vectors

These vectors were developed to enable the cloning of larger DNA fragments than could be handled in lambda and cosmid vectors. Just like real chromosomes, these artificial yeast chromosomes have two arms, and large DNA fragments can be ligated into both. The arms contain a selection marker and DNA sequences which can function as telomeres in yeast. In addition, one arm has sequences which can function as centromeres and as origins of replication. Recombinant YACs can be introduced into yeast and mammalian cells by transformation. The application of YACs and MACs (mammalian artificial chromosomes) is further discussed in section 5.1.3.1.

4. Strategies for gene transfer

Table 1 lists the most commonly employed strategies for transferring nucleic acids to living cells *in vivo* (in an intact organism) or *ex vivo* (in cell cultures or isolated organs), and mentions some of their important characteristics.

Genetically modified viruses have so far been the most important vectors for vaccination and genetic therapy. Over millions of years, viruses have evolved effective methods which ensure that the viral genes are expressed, often without damaging consequences for the cells. Foreign genes that are inserted into the virus genome will be expressed at the same level as the virus's own genes. The pox virus (vaccinia), retrovirus- and adenovirus-based vectors have been much used for gene transfer, and most recently the herpes virus and parvovirus (adeno-associated virus, AAV) vectors have also come to the forefront. All the virus vectors have their advantages and disadvantages as regards host cell and species specificity, capacity to accept foreign DNA, level of genetic expression, stable presence within, or introduction into, the organism, and possibilities for non-desirable influence on cellular functions. These aspects are discussed in detail in many recent review articles (Vogel, 1993; Levine & Friedman, 1993; Morgan & Anderson, 1993; Walsh *et al*., 1993; Gilboa & Smith, 1994; Yu *et al*., 1994) and will not be elaborated upon here.

Table 1

Methods for transfer of naked DNA to mammalian cells [1,2]

Application

Method	***Ex vivo***	***In vivo***	***Genetic expression***
Direct injection	+	++	**Transient**
Electroporation	++	**-**	**Stable after selection**
Calcium phosphate precipitation	++	**-**	**Stable after selection**
Liposomes	+	++	**Transient**
Ligand/DNA conjugates	**-**	++	**Transient**
Virus:			
Retro	++	++/?	**Stable**
Adeno	+	++	**Transient**
Adeno-assoc.	++	**?**	**Stable**
Vaccinia	+	++	**Transient**
Herpes	+	++	**?**

1 Modified from Lyerly & DiMaio (1993).
2 + little used; ++ much used; - not used; ? undergoing trials

The problems and deficiencies associated with the available virus vectors have necessitated alternative and complementary methods for the controlled introduction of naked nucleic acids into living cells and organisms.

Most of the methods that are employed are based on natural processes for uptake and intracellular transportation of macromolecules. Mammalian cells take up DNA by adsorption and/or specific vectors (Loke *et al*., 1989; Yakubov *et al*., 1989; Whitton, 1993; Stein *et al*., 1993). The DNA is then transported to the cell nucleus where it usually becomes *integrated* at random sites in the chromosomal DNA. Some of the internalised DNA may also, to varying degrees, be found as more or less disintegrated molecules in cytoplasmic vesicles.

Integration may be important for achieving a long-term effect from gene therapy since, for many types of vectors, this is a prerequisite for continuous expression of the therapeutic gene. On the other hand, integration can lead to changes in the genomic organisation and genetic expression of the receptor cells which may result in the development of cancer, developmental anomalies and metabolic illnesses.

It is in relation to just these problems that vectors based on adeno-associated viruses (AAV) are so attractive (Walsh *et al*., 1992). Such vectors integrate relatively specifically in a specific area on the human chromosome 19, whereas most of the other vectors apparently integrate randomly. However, whether this is really an advantage may be open to debate (Lyerly & DiMaio, 1993).

Gene transfer using the chemical and physical methods described in the rest of this chapter is unpredictable as regards integration. The types of vectors rather than the transfer mechanism probably determine the course taken. Successful gene transfer is often characterised by high, but temporary genetic expression from non-integrated DNA. The treatment has to be repeated, but because of simplicity, safety and reproducibility, the methods described will continue to be developed and applied (Lyerly & DiMaio, 1993).

4.1 Micro-injection and biolistics

Micro-injection into the nuclei of the individual cells has the advantage that DNA does not end up in cytoplasmic areas, where it would be broken down. The method cannot, however, be used on a large number of cells. Direct injection into mammals or fish may become widely used in connection with vaccination or immunisation (see Chapter 5), but also for local gene therapy performed by injection of nucleic acids into specific arteries and veins serving individual organs. Such therapy will also be relevant in the event of an illness affecting the blood vessels themselves. However, it is obvious that injected DNA will be spread throughout the organism and this may have unpredictable consequences.

The use of tiny gold balls with nucleic acids attached to them, which are shot through the skin (*biolistics*), will make direct injection simpler, more efficient and more widely used. Successful transformation of organs or cells using biolistics has been transferred to *in vivo* conditions, and DNA transformation and genetic expression have been achieved in the skin, liver and other organs of mice

(Williams *et al.*, 1991; Tang *et al.*, 1992; Fynan *et al*., 1993; Barry *et al.*, 1994). The technique has been recently modified enabling the use of equipment that is simple, cheap and commercially available (Vahlsing *et al*., 1994). Biolistic technology is sure to be adapted for gene therapy and immunisation in people. Internal organs exposed by surgery will also become available for gene therapy through this technology. However, it goes without saying that all the DNA will *not* end up where it is intended.

4.2 Electroporation

Nanometer-sized cell membrane pores are formed when mammalian cells, or bacteria, are exposed to short, high-voltage electric pulses (Keating & Toneguzzo, 1990). DNA in the form of, for example, an expression plasmid, is taken directly into the cell, either through the pores or because the membrane components which are supposed to close pores acquire a different form or distribution. The method can obviously only be used on cells in cultures.

4.3 Calcium phosphate precipitation

DNA which is co-precipitated with calcium phosphate is taken up by the target cells through endocytosis and is transported to the nucleus. Many established cell lines and bacterial strains are able to take up DNA efficiently following such treatment. However, primary cells within, or from, a mammalian organism have often proved to have low transfection efficiency when this method is used (Kato *et al*., 1986), which obviously limits its use in both gene therapy and vaccination contexts.

4.4 Transport of liposomes (lipofection)

Liposomes are spherical vesicles which vary in diameter from a few tens of nanometres to many millimetres. They consist of a fluid-filled vesicle surrounded by a continuous double layer of lipid molecules, generally phospholipids, analogous to the membrane of living cells. The composition of the lipid in liposomes may vary, likewise that of the fluid in the vesicle, thus giving liposomes with different properties (Smith *et al*., 1993). More and more effort is constantly being put into developing new forms of lipids (Felgner *et al*., 1994). The name *cytophectines* has now started to be used for this class of positively-loaded lipid particles which can improve the insertion of functionally intact macromolecules into living cells and organisms.

The use of liposomes as carriers for DNA and other nucleotides is really only a natural extension of their former application in chemotherapeutics (Stewart *et al*., 1992; Smith *et al*., 1993). Cationic liposomes have been used increasingly during the last 5 or 6 years to improve the transportation of DNA, mRNA, anti-sense oligonucleotides and proteins into living cells. Liposomes have been used *in vivo* (in animals used for experimental purposes) to deliver macromolecules systematically to catheterised blood vessels, epithelial lung cells, brain tissue and *Xenopus* embryos. Clinical trials of liposome-mediated gene therapy in people have already been carried out (Nabel *et al*., 1992).

A great deal of international research effort is now being mobilised to produce liposomes with predetermined target cell specificity. This will then be combined with enhanced resistance to breakdown and elimination within the organism carried out by cells belonging to the reticulo-endothelial system (RES), such as macrophages.

Enhanced target cell specificity can be achieved by what are called "*immunoliposomes*" (Wang & Huang, 1989). Such liposomes have covalently bound antibodies, and this is often combined with the liposome membrane being pH sensitive. The antibodies are directed against the surface structures of a particular type of target cell. After the antibodies have reacted with the surface structures, the liposomes are taken into the cellular endosomes. When the natural drop in pH takes place in these, the liposome membrane becomes destabilised and fuses with the endosome membrane, thus releasing its content, including the DNA, to the cytosol.

Liposomes have also been constructed which become unstable and empty themselves at temperatures in excess of 37°C. Such temperatures are common for certain types of cancer cells and can be induced from the outside in particular parts of the body. Both these and other ways of enhancing target cell specificity are undergoing trials in animals (see Hug & Sleight, 1991).

Attempts to solve the problem of the destruction of liposomes in RES have been made by, for instance, covering the liposomes with polyethylene glycol chains. These mimic the sialic acids which occur naturally on the surface of the red blood corpuscles. The liposomes will not then be recognised by macrophages and other RES cells, thus extending their half-life from a few hours to 2 days (see Needham *et al*. 1992 for a review). Monosialogangliocides or cholesterol have also been employed for comparable strategies see the review in Hug & Sleight, 1991).

As it will be apparent, a great deal of the research in this field is directed at making liposomes more resistant to naturally occurring inactivation mechanisms. The objective is obviously to increase the efficiency of gene transfer, but at the same time it will make liposome-nucleic acid complexes potentially more dangerous from an ecological viewpoint.

4.5 Ligand-DNA conjugates

This technique entails the setting-up of complexes between plasmid DNA and specific proteins or antibodies which can recognise receptors in the desired target cells. The internalisation routes in the cells are well known in the case of some cell-specific receptors of this kind, and it is possible to select routes which do not lead to lysosome-mediated degradation of the DNA within the target cells (Wilson *et al*., 1992; Hyde *et al*., 1993; Chen *et al*., 1994).

A general conclusion *regarding this survey of methods that can be employed to transfer naked DNA has to be that none of the methods can be carried out in ways which preclude the release of nucleic acids.*

5. Areas of use for naked nucleic acids

Until recently, genetic vectors were only used for transfection and transformation of cell cultures, a practice which now takes place on a very large scale in research laboratories and industrial concerns throughout the world. For some years, considerable attention has centred around the health and ecological risks associated with the release or escape of GM cells.

Most recently, the area of application has been extended to include the insertion of naked DNA into multicellular organisms, plants, animals and people. The volume of naked, genetically active DNA which is used, the areas of use and the chances of undesirable impacts on ecosystems through accidents or ignorance are increasing dramatically at the present time.

In the case of certain areas of application, such as vaccination or gene therapy, it is, in practice, impossible to prevent biologically active DNA from spreading away from the site where it was originally used. It is also an open question whether the laboratory procedures used in biotechnological manufacturing and biological research are sufficiently safe, but something can be done about this.

Attention has so far been directed at problems associated with the release or escape of living organisms or cells. We have become increasingly aware that phenotypically and genetically dead are two radically different states. A dead cell or organism will leave behind it nucleic acids which may remain biologically active for a long time.

In a given situation, the release of naked DNA may result in the spreading of unknown plasmids from or to GM micro-organisms released in connection with, for example, the breakdown of pollutants (Thiem *et al*., 1994). It is thus possible for *the properties of both naturally occurring and released micro-organisms to be changed in unpredictable ways* (see the reviews in Lorenz & Wackernagel, 1994 and Nielsen *et al*., 1998).

Usually, the application of naked DNA entails the transfer of double-stranded DNA which codes for a therapeutic protein, but rapid development in the use of anti-sense oligonucleotides and ribozymes (section 5.2), and the direct introduction of RNA into cells, gives rise to new, potential problems for health and the environment.

5.1 Gene expression from naked DNA in multicellular organisms

Like many other significant discoveries, this, too, happened by chance. What had prevented an earlier breakthrough was a dogma that naked DNA introduced to an intact animal organism would very quickly be broken down and would lack biological importance. The scientists who removed this dogma (Wolff *et al*., 1990) were really aiming to test chemicals which could raise the uptake of

naked DNA in the muscle cells of living mice. It concerned simple shuttle vectors used in laboratories all over the world. One of the obvious verifications in the trial protocol was that chemicals should be excluded, naked DNA alone being injected intramuscularly. It transpired that the muscle cells of these creatures efficiently took up DNA and produced larger quantities of the protein for which the plasmid coded than were found in the mice treated with chemicals. Naked RNA was also injected during the same trials, and those mice produced the absolute largest quantities of protein (Wolff *et al*., 1990), but that discovery, surprisingly enough, does not seem to have been followed up.

In the wake of this pioneering work, but also independent of it, a number of important studies were carried out which showed that genetic expression could be achieved after mice had been injected with simple expression plasmids containing genes with potential therapeutic application (Acsadi *et al*., 1991; Raz *et al*., 1993). The possibilities for directed genetic expression using such strategies was also demonstrated in several species of animals. For instance, it was shown that if adolescent *carp* (ca. 10 g) received an intramuscular injection of plasmids containing reporter genes (β-galactosidase, CAT) under the control of various promotors (including human, viral and rabbit), a powerful genetic expression was obtained (Hansen *et al*., 1991). Intravenous injection of naked, circular virus DNA into rabbits and mice gave a powerful genetic expression with the development of antibodies and the production of new virus particles (Fredriksen, 1993; Fredriksen *et al*., 1994).

For so far unexplained reasons, there seemed, in practice, to be major differences between the plasmids used for breakdown in the organism and/or uptake into various cells and organs. Only injection into musculature proved to give a reproducible genetic expression from naked DNA. However, it soon transpired that if mice were given intravenous injection of plasmids in liposomes (see Chapter 4), this was able to give expression in several organ systems (Zhu *et al*., 1993) including *the ovaries, certainly a highly undesirable result because a possible integration of plasmidic DNA in the chromosomes of the sex cells can lead to the inheritance of a genetic change. The same problems have now been highlighted in further gene therapy trials in animals as well as humans* (Boyce et al., 1998).

Subsequently, using intravenous injection, or local installation in the respiratory passages, scientists achieved *in vivo* gene transfer to rabbit lungs of a plasmid which contained the gene for recombinant human alpha l-antitrypsin, driven by a CMV (cytomegalovirus) promotor in complex with cationic liposomes. Both insertion routes gave expression in the lungs for at any rate 7 days (Canonico *et al*., 1994).

A careful study should be made to determine whether genetic expression from liposome-plasmid complexes following installation in the respiratory passages is a common phenomenon which can happen to plasmids that go astray. It is undesirable because such expression may lead to serious reactions in the respiratory passages.

5.1.1 DNA vaccination

When the major breakthrough for naked DNA in a vaccination context was published (Ulmer *et al*., 1993), it created a great deal of media attention. Ulmer and his coworkers were working on immunisation against the nucleoprotein (NP) for the Influenza A virus. DNA which coded for NP was inserted in an expression plasmid which gives protein production intracellularly under the control of either an RSV (Rous sarcoma virus) or a CMV (cytomegalovirus) promotor/enhancer. A couple of weeks after injection of plasmid DNA directly into the thigh muscles of mice, antibodies against the nucleoprotein were found indicating that the gene had been expressed. The mice were then infected with a dose of influenza which is lethal for non-immune mice. However, 90% of the DNA-vaccinated mice proved to survive the infection.

That the mice were immune was one important observation. However, at least as impressive was the cross protection attained against different strains of the Influenza A virus. The ever new variants that arise are, of course, one of the major problems associated with this virus in a public health context. Antibodies developed following infection or vaccination with one variant confer no protection against another (Cohen, 1993). Ordinary influenza vaccines, which consist of chemically-killed virus particles, do not provide cross immunity, probably because it is first and foremost antibodies against surface structures in the virus that are developed. It is these structures which vary from one virus strain to another.

The same group of researchers who reported the original influenza studies subsequently refined the DNA vectors, thereby achieving still better genetic expression and even more reliable immunity (Montgomery *et al*., 1993). Moreover, the same DNA vaccination strategy that was employed in the influenza studies has also achieved protective immunity against HIV 1 (Wang *et al*., 1993), Hepatite B (Davis *et al*., 1991) and Rabies virus (Xiang *et al*., 1994). A plasmid DNA which expressed the influenza virus, hemagglutinin, was tested with a view to achieving protection against lethal viral infections in mice and chickens (Fynan *et al*., 1993). This was partially achieved by intramuscular injections, intravenous inoculations and the use of aerosols containing a plasmid that were placed either in the nostrils or the trachea. However, by far the most effective immunisation was achieved using *biolistics*, small gold balls with DNA attached that were shot into the epidermis. This inoculation route could be used with amounts of DNA down to 0.4 mg, whereas the other routes required 50-300 mg. The same biolistic methodology, termed “genetic immunisation”, has been employed to produce both polyclonal and monoclonal antibodies against several types of antigens (Williams *et al*., 1991; Tang *et al*., 1992; Barry *et al*., 1994), and the method has subsequently been modified allowing simple, commercially available equipment to be used (Vahlsing *et al*., 1994).

Generally speaking, vaccines based on purified proteins from viruses or bacteria, produced conventionally or by genetic engineering, like killed virus particles, first and foremost produce antibodies (humoral immunity) against the virus, but give poor development of cyto-toxic T lymphocytes which destroy virus-infected cells (cellular immunity) (Ulmer *et al*., 1993; Wang *et al*., 1993; Xiang *et al*., 1994). The cellular immunity is broader than the humoral one, because the T lymphocytes overlook small differences between the virus strains, and for many viruses it has long been established that activated T lymphocytes stop or combat infection more effectively than antibodies.

Proteins which, with DNA vaccination, are expressed intracellularly from expression plasmids, are presented for the immune system in combination with tissue-type antigen class I molecules on the cell surface in exactly the same manner as takes place in a natural viral infection. DNA vaccination therefore gives the same type of immunity as that attained by undergoing a natural infection (Cohen, 1993).

Another advantage with DNA vaccination is that immunological defence is triggered against the protein(s) for which the plasmid codes, but not against the plasmid itself. Theoretically, this means that the plasmid vector can be used repeatedly to deliver different genes, thus conferring protection to several different infections (Cohen, 1993). Many people are now envisaging an "omnivax" vaccine which contains a cocktail of plasmids with genes from different pathogens, thereby providing protection from several infectious diseases.

When vaccination takes place with a simple plasmid vector, only the genes which one wishes to immunise against are expressed, whereas the use of viral vectors means that the individual will be given several other biologically active genes (Barry *et al*., 1994), and this is obviously, in principle, undesirable.

In contrast to recombinant proteins, which are produced in test tubes, proteins which are made by the animal itself following instructions given by the gene in the expression plasmid need not undergo extensive, expensive cleansing procedures (Cohen, 1993).

Relative to traditional vaccines which contain proteins, DNA vaccines are much less dependent on cooling chains, because DNA is more temperature resistant than proteins. This is a very important practical aspect in those parts of the world where vaccination is really most necessary.

Vaccines have to be cheap, safe and effective. Many people now believe that in a short time a number of DNA vaccines will be developed which will satisfy all the demands that can be made, and that they will become very widespread. Development has been so rapid that the New York Academy of Sciences held a 3-day conference in Arlington, Virginia already in April 1995 which was solely concerned with naked DNA vaccines (DNA vaccines: a new era in vaccinology). In the years that have followed, the prospect of DNA vaccination has given high hopes for immunisation against notorious killers like HIV (Letvin, 1998), various forms of cancer (Benton & Kennedy, 1998) and malaria (Hoffman *et al*. 1997).

It has not been conclusively determined whether the various plasmid vectors that may be used can become integrated in the chromosomes of the planned or accidental target cells. The integration tendency will probably vary for the same plasmid in different target cells and for different plasmids in the same type of target cell.

5.1.2 Vaccine-based therapy

Some investigations, trials and data exist which indicate that vaccination can be used not only to prevent, but also to treat infectious diseases. Animal trials and limited clinical trials have in some cases demonstrated dramatic, positive changes in symptoms and courses of illness (e.g. Nesburn *et al*., 1994; Benton & Kennedy, 1998). Steps are now being taken to clarify the therapeutic effect of such superimmunisation. A number of major, controlled trials are currently being performed involving vaccination of people with herpes infections, leprosy, tuberculosis, leishmaniasis and hepatite B (Cohen, 1993). If a positive effect can be documented for these, and perhaps also other infectious diseases, the total need for, and use of, effective vaccines will greatly increase. In that case, this tendency will be strengthened by the need for alternatives to antibiotic treatment, since the problem of resistant bacteria will only become more serious and effective therapeutics against viral infections and cancer will still not exist.

If naked DNA vaccines prove as advantageous as preliminary results imply, the use of naked DNA and the proportion of it which ends up in the wrong place will necessarily increase greatly in the years to come.

5.1.3 Gene therapy

In simple terms, gene therapy can be defined as *transfer of new genetic material (DNA or RNA) to the cells of an individual, with a consequent therapeutic advantage for the individual* (Morgan & Anderson, 1993). *Double-stranded DNA* will usually be transferred, but rapid developments in the application of *anti-sense oligonucleotides*, *ribozymes* and *direct introduction of RNA* necessitate a broad definition. The objective of gene therapy will often be to introduce a gene that codes for a therapeutic protein which the organism has a wrong version of, does not produce, or produces wrong amounts of, but it may also be appropriate to prevent the expression of harmful genetic products.

The broad definition says nothing about whether cells removed from the patient are treated and then replaced, or whether cells are treated *in situ*, within the intact patient. Nor does the definition distinguish between genetic and other kinds of disease. It can be envisaged that the protocols can be employed to treat such varied kinds of disorders as cancer, cardiovascular diseases, metabolic illnesses and infectious diseases, such as AIDS. Gene therapy can revolutionise medicine and veterinary medicine. *Any illness in humans and animals may, without exception, be understood as changes in the expression of genes, and is therefore, in principle, available for gene therapy (Levine & Friedman, 1993). It can otherwise be envisaged that gene therapeutic methodology will be used to supply, or alter, any genes whatsoever, thus introducing both ethical and ecological challenges.*

The *acceptance* of gene therapy as a clinical practice for people is taking place more rapidly than anyone dreamed of (Levine & Friedman, 1993). The technology and concepts behind this rapid development have their background in the studies of RNA and DNA tumour viruses in the early-1970's. The discovery that such viruses stably supplied the genetic material of the host cell with genes sowed the seed for the idea of using them to transfer genes. The first article to discuss the

possibility of using gene therapy to treat human illness was written by Friedman and Roblin and is dated in 1972. Their conjectures came to appear more realistic when the development of recombinant DNA technology enabled the routine isolation, manipulation and transfer of genes towards the end of the 1970's. This was followed by the development of retrovirus-based gene transfer methods in the early '80's. These demonstrated that genes *could* be extremely effectively transferred to both cell cultures and animals. Development has gone still faster since then. The first real gene therapy experiment was carried out in 1990 (review in Morgan & Anderson, 1993), and in 1998 alone more than 300 reviews concerning gene therapy have been published (Medline, 23 November 1998).

Today, many tens of gene therapy protocols have been approved for use on people, and new ones are steadily being added (Levine & Freidman, 1993; Boxhorn & Eck, 1998; Wivel & Wilson, 1998; Favrot et al., 1998). What still prevents a wider distribution and routine application of gene therapy are technical difficulties associated with:

i) efficient introduction of nucleic acids into some types of cells
ii) long-term maintenance of therapeutic levels of genetic expression
iii) possibilities for undesirable side effects of the integration of foreign DNA into the cell chromosomes. *Integration can lead to the cellular genes becoming steered from control elements in the introduced DNA, cellular genes being destroyed, or fusion proteins being constructed from the polypeptides and peptides of the cell, itself, coded for by the added DNA. The results can be registered as everything from cell death, via metabolic disturbances to development of cancer.*

These problems and the entire status of gene therapy are discussed in a number of recent, general (Morgan & Anderson, 1993; Levine & Friedman, 1994; Wivel & Wilson, 1998) and specialised (Vogel, 1993; Walsh *et al*., 1993; Gilboa & Smith, 1994; Yu *et al*., 1994; Dilber, 1998; Boxhorn & Eck, 1998; Resnikoff & Conrad, 1998; Favrot et al., 1998) review articles, and will not be commented on further here.

The general preconditions for gene therapy consist of <u>effective transfer</u> and <u>optimised expression</u> of a gene or genes (Wivel & Wilson, 1998). An essential step in all gene therapy will be to have the relevant gene transported to the correct organ and into the correct target cells (see Chapter 4). Despite great advances, several important technical problems in optimising gene transfer and gene expression remain. Such technological development will be decisive for how widespread the clinical use of gene therapy can become (Lyerly & DiMaio, 1993; Wivel & Wilson, 1998).

Gene therapy can have several objectives:

i) introduce genes which code for desirable proteins
ii) produce RNA with desirable functions (e.g. anti-sense and ribozyme)
iii) destroy genes which code for undesirable and harmful products.

Practical gene therapy can be performed either ex vivo or in vivo (Lyerly & DiMaio, 1993). *Ex vivo* means that the target cells are removed from the organism and supplied with the desired genetic material. The cells can undergo some form or other of selection to increase the proportion of cells which have taken up DNA or RNA, and then be put back into the organism. This strategy has a

number of *important advantages*: the methods used for transfer are more effectively applied on cells in culture than in an intact organism, cells which have taken up the desired DNA or RNA can be selected, the genetic expression can be measured, and the treated cells can be investigated for undesirable properties before being returned. *The problems* rest in, for example, the procedures for removing the target cells, the conditions for cell culture and the subsequent return of the treated cells.

In vivo gene transfer, directly within the intact organism, may seem less complicated. However, reliable, reproducibly efficient *in vivo* gene transfer has so far not been achieved. An important limitation lies in getting the DNA vector or oligonucleotide selectively transported to the correct target cells. This problem is now being attacked experimentally using methods for attaching nucleic acids to carrier molecules (see Chapter 4) and by developing new transfer vectors (see the next section). Experiments are also being performed in the direction of gene therapy on whole, extracted organs prior to autologous, allogenic or xenogenic transplantation (Tomita *et al*., 1992). Furthermore, xenotransplants from genetically modified animals (e.g. pigs) are now coming within reach (Fung *et al*., 1997), although both problems related to immunological mechanisms and potential for transfer of endogenous viruses represent serious obstacles (Borie *et al*., 1998).

5.1.3.1 Mammalian artificial chromosomes (MACs)

To achieve a permanent biological and/or therapeutic effect from the transfer of DNA requires (Huxley, 1994):

i) effective transport into the target cells
ii) long-term presence of inserted DNA
iii) physiological level and verification of the expression of the introduced gene.

The correct level of physiologically verified genetic expression in mammals can be most reliably achieved following the transfer of *intact genes in DNA fragments which have a size of several hundred kilobases*. This has been demonstrated by the transfer of YACs (yeast artificial chromosomes) to transgenic mice (review in Huxley, 1994). Long-term maintenance was achieved if the DNA had a replication origin, a centromere and a telomere which permitted segregation in mammalian cells. All these elements should theoretically be capable of being combined in a **mammalian artificial chromosome (MAC)**. The purpose of MACs is to construct vectors which mimic the chromatin structure of the mammalian genome, where introns and long-range elements are required for correct expression. MACs should be able to be maintained in mammalian cells alongside the endogenous chromosomes, without integration, and provide physiological expression of a gene in any kind of cell. Methods which will permit the transfer of such large DNA fragments include liposomes and receptor-mediated uptake. Both methods of transfer have proved to be able to function *in vivo*, and this has meant that such large constructs as MACs are believed to be potentially usable in a therapeutic context. It is assumed (Huxley, 1994) that since viral sequences are not involved, there is neither any possibility for recombinations with wild types of virus or immune reactions with viral proteins, nor any risk of insertion mutagenesis since MACs are constructed to be incapable of being integrated into the cell genome. Recently, very promising results have been

obtained by genetic trimming and engineering of the X chromosome (Farr *et al*., 1995) and by insertion of human centromeres and other elements into YACs (Ikeno *et al*., 1998).

In transgenic mice, the presence of procaryotic vector sequences and the lack of introns have been found to significantly retard genetic expression. It was also found that to permit full genetic expression, non-coded verification units, hypersensitive sites for DNAs, had to be present up to 65 kbp upstream or downstream from the transgene (Grosvold *et al*., 1987; Higgs *et al*., 1990; Carson & Wiles, 1993). The control sequences bind tissue or development-specific transcription factors which are also bound to the enhancers that are immediately associated with the gene and increase the gene expression in both the cultures and transgenic mice (Huxley, 1994).

YACs represent an ideal means of cloning DNA fragments that are sufficiently large to include human genes with all associated long-range acting control elements, whether they be located upstream, downstream or in introns (Huxley, 1994). However, a major problem is that YACs integrate in the host cell chromosomes. Such constructs in transgenic mice have proved to give tissue-specific expression on the same level as the cell's own genes following integration in host cell chromosomes. Even the 400 kbp large precursor amyloid protein gene was fully expressed on a level corresponding to the organism's own genes in the brain, heart, kidneys and testicles of transgenic mice following the transfer of a 650 kbp YAC (Lamb *et al*., 1993). These results on mice which carry intact YAC DNA indicate that transgenes on genomic fragments of moderate size are often expressed at low and variable levels, whereas transgenes carried on fragments which measure hundreds of kilobases become fully expressed (Huxley, 1994). However, it is not clear whether the same DNA motives will be necessary for a high expression level if DNA is maintained as an extra chromosome instead of becoming integrated. With the exception of Epstein-Barr virus (EBV) based plasmids, DNA is usually only temporarily maintained in cell cultures if it is not integrated, and even though promotor and enhancer elements are needed for high expression levels, long-range elements are not required.

Here, as is often the case otherwise, an unforeseen, and for the time being unexplainable, difference transpires between cell cultures and intact animals.

Relatively long-term expression from non-integrated DNA is achieved *in vivo* following injection into muscle cells, intravenous injection of liposome-complex DNA, or particle bombardment in tissue where the turnover of cells is sluggish (Wolff *et al*., 1990; Cheng *et al*., 1993; Zhu *et al*., 1993).

Transfer

DNA fragments which contain intact human genes and provide a full level of expression are often hundreds of kilobases. It is not easy to handle and transfer such DNA effectively and in an intact form, either *in vitro* or *in vivo*. The virus vectors, which perform the packing of DNA, have an upper limit ranging from approximately 10 kbp for retroviruses to approximately 150 kbp for pox viruses. Even the largest ones are thus still too small for such genes as the cystic fibrosis transmembranic regulator gene which spans over 230 kbp.

The methods which impart most hope and which, moreover, do not have any theoretical limitations regarding the size of DNA fragments that can be used *in vivo*, are particle bombardment (Cheng *et*

al., 1993), cationic lipides (Stribling *et al*., 1992; Zhu *et al*., 1993) and receptor-mediated uptake (Gao *et al*., 1993*).*

Maintenance

Virus genomes have mechanisms at their disposal for retaining viral DNA when that has been introduced into the cell. For retroviruses, and many others, this implies *integration* in the genome. For other viruses, such as EBV, the virus genome is maintained as extra-chromosomal, plasmid-like DNA elements. Non-viral DNA usually does not have these mechanisms. The fate of such DNA has been closely studied in cell cultures. Irrespective of the kind of transfer or transfection method employed, only a small portion, perhaps 1%, of the cells that take up and express DNA will really integrate DNA into their genome and become stably transformed (Huxley, 1994). Non-integrated DNA is rapidly lost from the cell culture.

Only a few systematic studies have been carried out on the maintenance and expression of DNA that is introduced into intact mammals. Naked DNA which is injected directly into the musculature enters cells and is expressed. DNA is not integrated and does not seem to replicate, but it is maintained and can be expressed, at any rate for one year (Wolff *et al*., 1990). This may reflect special properties in the muscle cells. Injections of the same kind into the liver, spleen, skin, lung, brain and blood gave only low levels of expression for the reporter gene which the plasmid contained. In the case of epithelial cells in the lungs, DNA is able to be supplied by inhalation of an aerosol of DNA in liposomes, and DNA is expressed at any rate for 21 days (Stribling *et al*., 1992). As regards other tissues in the body, DNA can be introduced by intravenous injection of liposomes. Most of the DNA is not integrated, but is retained and expressed in many of the cells in the lymphatic nodes and spleen for at least 63 days, and in the lungs for 150 days or more (Zhu *et al*., 1993). In the epidermis, liver and pancreas, the expression sank to 1-5% of the peak level after 1 week, whereas a stable level was maintained in the muscle cells for at least 1.5 years.

The long-term maintenance of DNA in the musculature, skin and lung tissue *in vivo* may be a result of the sluggish turnover of cells in these tissues. In that case, long-term expression will also be achievable in other tissues where cells exist which remain alive for a long time, for instance in the brain, provided DNA can be introduced *in vivo* (Huxley, 1994). In these tissues, therapeutic expression of genes from extrachromosomal DNA can be achieved with DNA which lacks special sequences for replication and segregation. However, it can be expected that integrated DNA will be lost from cells that are dividing, including parental stem cells, and it is into these it may be necessary to introduce therapeutic DNA in order to achieve expression that lasts significantly longer than the turnover speed of the cells in a special tissue. In parental stem cells, this must include some way of maintaining non-integrated DNA if that is not to be lost from the cells (Huxley, 1994).

Thus, a great deal of research is being directed at increasing the stability and survival of expression vectors. These are obviously goals that are important in a therapeutic context. However, as such targets are reached, the resulting DNA vectors will also have a potential for more serious ecological impacts when they escape and come into contact with non-planned target organisms or cells.

Replication

Viral constructs, which have mechanisms for DNA replication and extrachromosomal presence, illustrate how effectively DNA can be maintained if these functions are induced. For example, plasmids which carry the EBV replication origin (oriP) replicate efficiently in human cells where the EBV protein, EBNA 1, is also expressed, and can give rise to stably transformed cell clones containing extrachromosomal DNA. Plasmids containing oriP give 10-100 times higher colony figures than plasmids that are without it, and the number of stable transformants approaches the total number of cells which take up DNA. It seems that oriP contributes with functions for both DNA replication and for preventing loss from the nucleus (Krysan *et al*., 1989). These are desirable properties in a gene therapy context. However, like all viral replication and segregation systems, the oriP function requires a viral protein (in this case EBNA 1), and this may affect cellular functions in undesirable ways. *An alternative will then be to make use of the mammalian chromosomes own, natural systems for replication and segregation*. The problem, nevertheless, is that mammalian chromosomal replication origins are still not usable, despite intensive research. Replication origins have been found on mammalian chromosomes, but the DNA fragments which carry them do not usually form extrachromosomal elements when they are transfected into mammalian cells. This may be because they can temporarily undertake replication, but do not have the mechanism or mechanisms for locating the nucleus (Hamlin *et al.,* 1992; Caddle & Calos, 1992).

YACs are capable of being transferred to mammalian cell cultures where they can duplicate and be maintained for a time as extrachromosomal elements, but they segregate poorly during cell division and will therefore rapidly disappear (review in Huxley, 1994). However, this problem may be solved by inclusion of human centromeres and other human elements (Ikeno *et al.*, 1998).

Mammalian centromeres and segregation of therapeutic DNA

Segregation of daughter chromatids to new nuclei when cell division takes place depends upon the activities of what is called the kinetochore, which attaches itself to microtubuli in the mitotic spindle. Just what characterises DNA that has kinetochorous and centromeric functions is not known in detail, but some advance has been made as regards defining the delimitation of the centromere, cloning of centromeric DNA and isolation of centromeric proteins (reviews in Huxley, 1994; Farr *et al*., 1995; Ikeno *et al.*, 1998).

Telomeres and prevention of integration

If a DNA construct that carries a functional centromere integrates in host cell DNA a dicentric chromosome will be formed which will cause lability, including mutagenic occurrences. *One of the functions of telomeres, which are located on the ends of mammalian chromosomes, is to prevent free DNA ends entering into recombination processes.* Telomeres could thus be envisaged as preventing the integration of MACs following introduction *in vivo*. Human telomeric DNA consists of about 5 kbp of repeated TTAGGG sequences. Short stretches of this sequence are sufficient to achieve the formation of telomeres following introduction of linear DNA molecules into immortalised, cancer cell-like mammalian cell lines, but in parallel trials this is never observed in primary, normal human fibroblasts. *This probably shows, yet again, how difficult it is to generalise, and indicates that telomere sequences would not confer protection from integration in somatic tissue.* An alternative would be circular MACs which did not contain free DNA ends, since circular chromosomes are quite stable *in vivo* (reviews in Huxley, 1994; Farr *et al*., 1995; Ikeno *et al.*, 1998).

The conclusion to all this must be that our understanding of the properties that are necessary for replication and segregation of chromosomal DNA in mammalian cells is still not sufficiently complete to construct and use a MAC for therapeutic purposes. Even though such a construct would be a new, powerful tool in gene therapeutic contexts it would, at the same time, represent a potential risk for health and the environment.

5.2 Practical use of anti-sense nucleotides and ribozymes

Sequence-specific inactivation or destruction of nucleic acids without affecting normal host cell functions will theoretically be an ideal strategy for treating illnesses, such as infections and cancer, in fish, domestic animals and people (Akhtar, 1998; Rossi, 1998; Woolf, 1998; Kronenwett & Haas, 1998).

Anti-sense technologies are now being employed for specific downward regulation of undesirable genetic expression (Whitton, 1994). The principle consists of oligonucleotides being synthesised that are complementary to, for example, the sequence in a given mRNA. If such oligonucleotides are inserted into cells, they will hybridise with the mRNA. Under certain conditions, such hybridisation will lead to the mRNA being incapable of being translated, i.e. the protein for which the mRNA codes will not be produced.

This field was opened up 14 years ago (Mizuno *et al*., 1984) when naturally occurring translational retardation mediated by complementary (i.e. anti-sense) RNA in a procaryotic system was demonstrated. The system subsequently proved capable of manipulation in that the introduction of a synthetic, complementary RNA sequence blocked the production of the relevant protein in *E. colli* (Coleman *et al*., 1984). About the same time, it was shown that the strategy could be used for mammalian cells in culture. The introduction of RNA which was complementary to cellular mRNA from several genes suppressed their translation (Izant & Weintraub, 1985).

5.2.1 Advantages of the anti-sense strategy

The theoretical advantages relative to current treatment strategies (Whitton, 1994) will include: i) the complete target-sequence specificity that can be achieved minimises the risks of unforeseen toxic effects on the host cell or organism; ii) for infectious diseases, several anti-sense molecules can be employed, enabling several essential genes in micro-organisms or viruses to be hit simultaneously; iii) reduced risk of developing therapy-resistant variants of the virus, which, as is well known, is already a huge problem in the case of bacteria, but is also beginning to be so for viruses, especially in the form of chemotherapy-resistant Herpes simplex and HIV strains.

The specificity can be varied with the length of the anti-sense chain (Whitton, 1994). Theoretically, a 16-base sequence will be expected to occur once every 4^{16} bases, i.e. once per haploid mammalian genome of about $3x10^9$ bases. However, as only approximately 1% of the mammalian DNA (around 70,000 genes) is expressed at the RNA level (Bird, 1995), one oligonucleotide of 14 bases should be expected to occur on average once per mammalian RNA pool. To avoid affecting the host cell

functions, an anti-microbial, anti-sense nucleotide should have at least 14, preferably more than 16, bases. These calculations, however, ignore the fact that stable hybridisation may be taking place between complementary nucleic acids which have mismatches in 1 or 2 positions. Experience with the mRNA differential display technique indicates that in many cases full homology for 6 of 10 nucleotides may be sufficient for biologically meaningful hybridisation (Liang *et al*., 1993). However, it is conceivable that the specificity in some cases is greater than the theoretical calculations would imply, because some anti-sense oligonucleotides have proved capable of discriminating between target sequences which are differing at a single base (Saison-Behmoaras *et al*., 1991). *It is, however, not known what characterises these systems or how universally such high specificity can be achieved. What is certain is that unforeseen complementarity between anti-sense oligonucleotides and nucleic acids which are found in planned or fortuitous host cells may have terrible consequences.*

5.2.2 Available anti-sense strategies

There are three possible approaches (Whitton, 1994), which have in common that the specificity is achieved through complementarity between nucleic acid sequences:

1. ribozymes: catalytic RNA molecules which cleave other RNA chains in a sequentially-determined manner;
2. synthetic anti-sense, oligonucleotides (usually DNA) which are introduced to the target cells from the outside;
3. anti-sense RNA synthesised from a DNA vector which first has to be introduced into the target cell.

The last two represent the now almost "classical" strategies. They are distinguished by the chemical structure and manner of introduction of the effector molecules, and can affect gene expression at several levels: DNA synthesis, transcription and processing of RNA, reduced translation from RNA and enhanced RNA degradation. At best, the effector molecules act with a 1:1 stochiometry, i.e. each of them inactivates a target molecule. The ribozymes, on the other hand, offer the extra bonus that one effector molecule can destroy many RNA target molecules. When a target molecule is degraded, the ribozyme can base pair itself with, and thereafter destroy, a new one.

5.2.2.1 Ribozymes

After being discovered in the protozoan *Tetrahymena thermophila* (Kruger *et al*., 1982), naturally occurring ribozymes have now been found in plant-viroid and plant-virusoid RNA, RNA transcribed from special "satellitic" DNA in a species of salamander, and the human hepatitis B virus (Whitton, 1994).

The ribozyme forms which are most used in anti-sense strategies have been given names after their secondary structures and are therefore called *hammer-head* and *hairpin* ribozymes. So far, it is the hammer-head ribozymes that have been most studied with a view to therapeutic applications. However, the two kinds of ribozyme have different properties and both will probably find their uses,

sometimes perhaps even in combination (Whitton, 1994). Hairpin ribozymes may sometimes more easily gain access to enclosed target sequences, are more RNase-resistant and have greater relative efficiency (Kikuchi & Sasaki, 1991).

Ribozymes have two functional regions (Whitton, 1994): i) a catalytic "core" which cleaves target RNA; mutations in this destroy the activity of the ribozyme, and ii) flanking regions which, on the basis of complementarity, steer and place the core in a position to cut a specific site in the target RNA. It is therefore possible to construct and synthesise ribozymes with flanking sequences which steer the ribozyme to a predetermined cleavage site. Such a site typically consists of only 2 or 3 specific bases (e.g. 5`GUX 3`or even only 5`UX 3`, where X may be any base whatsoever). Cleavage sites will therefore occur very frequently in RNA molecules and will be found arbitrarily with a frequency of 3/16.

Introduction methods

Ribozymes can, in principle, be introduced into cells or organisms in three different forms: i) as ready-made, pre-synthesised RNA molecules, ii) in a DNA expression plasmid, iii) packed in a recombinant virus. The first two cases concern transfer of naked DNA or RNA.

Most examples of ribozyme effects in cell cultures have been based on molecules coded in DNA (virus or plasmid) which have been transcribed in the target cells. Few reports exist of the introduction of pre-synthesised RNA molecules. However, if ribozymes are to become therapeutic tools it would be advantageous to have a complete menu of possible introduction methods, including pre-produced molecules, even though these would be tremendously sensitive to nuclease-determined breakdown in an organism. Attempts have been made to change this by chemical modifications of the ribozymes, and many modifications are at present being investigated (review in Whitton, 1994). It has already transpired that some hybrid ribozymes with RNA core sequences and DNA flanking sequences have increased stability.

Yet again, it must be pointed out that increased stability is desirable from a gene therapy viewpoint, but it may lead to the risk of ecological impacts increasing.

Therapeutic use of ribozymes

In cell culture experiments, the possibilities for therapeutic use have been demonstrated for virus infections like HIV (Lo *et al*., 1992), lymphocytic choriomeningitis virus (Xing & Whitton, 1993) and Dengue virus. The last-mentioned is the cause of one of the most important infectious diseases in the world, with about 100 million cases of illness annually (Raviprakash *et al*., 1995). Furthermore, the potential for treating cancer is under continuous investigation (Scanlon *et al*., 1991; Kashani-Sabet *et al*., 1992; Koizumi *et al*., 1992). Transport into culture cells of pre-produced ribozymes with specificity for TNF-a RNA was achieved with the help of liposome-mediated transfection. This led to an approximately 90% reduction in the intracellular quantities of TNF-a mRNA and protein (Sioud *et al*., 1992).

5.2.2.2 Classical anti-sense strategies

According to Whitton (1994), these concern the use of: i) oligo DNA nucleotides (*ODN*) as pre-formed effector molecules, ii) anti-sense in the form of sequences on a DNA expression plasmid, iii) oligo RNA nucleotides - analogous to ODN.

There are good review articles about the use of anti-sense ODN (see e.g. Stein, 1992). Both standard ODNs and chemical modifications of them have been used. Low stability in cell cultures and living host organisms have proved to be a problem because such short chains will be very prone to nuclease breakdown. A great deal of work has therefore been expended on stabilising the ODN molecules in such a way that their ability to hybridise with target sequences is not diminished. For instance, to enhance their stability, non-bridge building oxygen atoms in a phosphate group have been replaced by either sulphur, selenium or methyl. When other modifications have been carried out, attempts have been made to simultaneously raise both stability and uptake into target cells, for example by conjugation to polylysin or cholesteryl (review in Whitton, 1994). The most recent development is peptide-nuclein acids (PNA) which involve nucleoside units carried on a polyamide backbone. These are capable of invading duplex DNA and causing the formation of a *D loop*. Micro-injection of a PNA of 15-20 residuals in the nuclei of the target cells resulted in a sequence-specific downward regulation of the genetic expression (Hanvey *et al*., 1992).

We see that regarding ODN, too, intensive research is taking place directed at modifications which make anti-sense molecules more dubious in an ecological context.

ODN is generally directed at mRNA, but there are also examples of the target being DNA (Whitton, 1994). When the target sequences are DNA, the situation is exploited that pyri-midine oligonucleotides, under certain conditions, can attach themselves in major grooves to double-stranded DNA, thereby forming a local triple helix. Adhesion of a photo-activat-able psoralene conjugate to either the 5`, or 3` end of the ODN then gives the opportunity for cross-linking of the two original DNA strands following hybridisation of the ODN psoralene complex (Perrouault *et al*., 1990). The target DNA will thus be rendered biologically inactive.

It will have become apparent that such constructs have relatively little specificity and must be looked upon as being very dubious from the points of view of the environment and health.

When the target sequences are RNA, anti-mRNA ODN can achieve an effect in at least two ways:

i) By physical blocking. An ODN which is complementary to the ribosome attachment site on an mRNA will prevent translation being initiated, whereas one which attaches further down can prevent the polypeptide chain from being extended. The effect will be stochiometric: one oligo can inactivate a single target molecule.
ii) An ODN-RNA hybrid will be sensitive to the target cell's own nucleases. RNase H will, for example, degrade the RNA portion of the double strand, destroy mRNA and liberate the oligo for attachment to another mRNA molecule. Hence, this strategy entails, in common with the ribozymes, recirculation of the effector molecules. The target sequences on the mRNA may vary; both ribosome attachment sites, initiating codes for translation and donor/acceptor sites for splicing (Kulka *et al*., 1993; Raviprakash *et al*., 1995; Caselmann *et al*., 1997) have been

used with success, as also have oligos that are complementary to three non-translated sequences on the mRNA (Raviprakash *et al*., 1995). Target sequences within the protein coding portions of mRNA have given extremely variable results (Whitton, 1994).

The mechanisms for uptake of ODN in living mammalian cells are poorly understood (Whitton, 1994). The attachment level for the cell membrane varies greatly from one type of cell to another, and is not even constant for a given type of cell. Both infection with a retrovirus and stimulation of certain intracellular signal routes can increase the attachment of oligonucleotides to the cell surfaces (Kitajima *et al*., 1992b).

The uptake of ODN depends on both time, temperature and concentration, and it consumes energy. Some data strongly indicate that a *specific cellular receptor*, an 80 kD protein, is responsible for the attachment of oligonucleotides and their internalisation by endocytose (Loke *et al*., 1989; Yakubov *et al*., 1989; Vlassov *et al*., 1994). In other types of cell, the binding and uptake seem to be less specific, the oligonucleotides being treated as toxins which are rapidly removed from the environment, and the intracellular concentration will become a balance between uptake with non-specific pinocytose and liberation with active exocytose (Stein *et al*., 1993).

Irrespective of the mechanisms that lie behind it, it remains certain that oligonucleotides can gain access to intracellular space, and that high concentrations of oligonucleotides are cytotoxic. Several variables exert influence at the lowest oligonucleotide level which causes the cytotoxicity: the length of the oligonucleotide chains, the base composition, chemical modifications (diester, thioate, methyl phosphonate, etc.), how the oligonucleotides are introduced, the type of cell, the cell culture conditions, etc. (Crooke *et al*., 1991; Caselmann *et al.*, 1997), but few systematic studies have been made in this important field. The therapeutic index for anti-sense ODN is often low. *Cellular toxicity may be a problem already at a concentration that is 4-10 times higher than what is necessary for a therapeutic effect* (Whitton, 1994; Akhtar, 1998; Branch, 1998).

As regards *supply of anti-sense RNA to target cells*, it has been reported that pre-formed RNA in antibody-targeted liposomes has been employed with success (Renneissen *et al*., 1990). However, it has been usual to get anti-sense RNA expressed from DNA templates introduced by transfection or infection. Many plasmids and virus vectors have been tried. According to Whitton (1994), adeno-associated virus (AAV) may prove especially useful (Chatterjee *et al*., 1992).

Anti-sense oligonucleotides in living organisms

The ultimate objective for anti-sense studies is obviously to be able to employ the technology to solve relevant problems (Branch, 1998).

When it comes to *non-mammalian systems*, a number of examples have been reported in the literature of transgenic plants that have had their disease resistance, maturing time, etc. changed as a consequence of anti-sense expression. Dramatic effects have also been observed in *Drosophila*, and some of the very first studies of anti-sense expression were carried out on *Xenopus laevis*. It can safely be concluded that there is good documentation that anti-sense molecules can produce biological effects in non-mammalian eucaryotic organisms (Whitton, 1994; Akhtar, 1998; Woolf, 1998). *In that case, it may be all the more serious if anti-sense oligonucleotides employed for therapeutic purposes escape.*

As regards living mammals, experiments with anti-sense expression in transgenic mice have often underlined the problems that may be met and the unpredictability in a system where so few of the variables are understood (Whitton, 1994). *This is, incidentally, a good rule to remember for the whole of this report!*

However, it must nevertheless be emphasised that some interesting and promising results have been achieved. Anti-sense inhibition of G proteins led, for example, to developmental defects in the target organs (Moxham *et al*., 1993), and virus-induced leukaemia was prevented by expression of anti-viral, anti-sense RNA (Han *et al*., 1991). Mice which expressed a transgenic coded C-MYC protein and were injected intravenously with 300 mmol anti-sense methyl phosphonate-ODN showed a reduction in c-myc mRNA and in MYC protein levels (Wickström *et al*., 1992). Virus-transformed tumour cells transplanted into mice grew rapidly and were lethal within 8-12 weeks, However, if anti-sense ODN against mRNA for the transcription factor NFkB was given up to 7 days after the tumour transplantation, the cancer growth was reduced and the mice survived. The ODN was phosphonothioate-modified and was given by interperitonal injections of 40 mg per gram of body weight every 3rd day, with a total of 3 injections (Kitajima *et al*., 1992a).

Such results obviously necessitate further research with the aim of being able to treat infections and cancer in people, and it would be unethical not to do so. However, methods and procedures for avoiding ecological side effects should go hand in hand with the therapeutic experiments.

6. Persistence of naked nucleic acids in Nature

6.1 Free DNA in the environment

This is a key problem in connection with pre-assessments of damaging effects and includes not only the power of resistance of nucleic acids generally, but also the length of fragments which can persist for how long under what conditions. Even though this may seem very simple put like this, it is a very complex field of research. Specific properties in the nucleic acids concerned clearly play a major role, and in addition the field covers mutual impact between a number of freely varying environmental factors (for a recent review, see Nielsen *et al*., 1998).

The new branches of science, *molecular palaeontology* and *molecular archaeology*, show quite clearly that relatively long chains of chromosomal DNA can survive for a long time under certain conditions. Even leaving aside Jurassic Park, 65 million years, etc., there is proof of survival over thousands of years (Pääbo *et al*., 1988). Controlled biochemical studies concerning the breakdown of DNA in solution under "normal" conditions imply that DNA generally will be severely degraded, if not totally broken down, after 40-50,000 years (reference in Morell, 1993). *However, it is, as we know, one of the inherent curses of science that Nature only seldom views it as a priority to reproduce or mimic "normal" laboratory conditions!*

When it comes to survival of DNA under *natural* environmental conditions, on the whole little research has been done in this important field. Moreover, most of the reported trials have used pure, homogeneous clay and sand as the DNA recipient, and these have completely different properties from the far more heterogeneous and complex, naturally occurring soils. This is illustrated by a recently published study (Ogram *et al*., 1994) which shows the dramatically differing extents in which varying lengths of DNA fragments (from about 2 to 23 kbp) can be adsorbed by various types of soil. This work also very convincingly demonstrates how adsorption to solid surfaces can have major consequences for DNA survival, because different types of soil particles give varying degrees of protection from DNase attack. Even the largest fragments, under favourable circumstances, could be recovered intact after several weeks. Romanowski *et al*. (1993) also showed that the type of soil is important and demonstrated the continued existence of transformable plasmids 60 days after release. Recorbet *et al*. (1993) demonstrated the persistence of substantial quantities of chromosomal DNA from genetically modified *E. coli* 60 days after the bacterial culture had been inoculated in natural soil. This was much longer than bacterial cells could be detected by plate counting and immunofluorescence. Widmer *et al*. (1997) found persistent transgenic plants from tobacco and potato marker gene *nptII* in soil for 77-137 days.

Free DNA has been found in all the ecosystems (sea water, fresh water, sediments) so far investigated (Lorenz & Wackernagel, 1994), even though DNases are widely distributed. Pooled data acquired by various methods show that such DNA is present in significant amounts, most of it having a microbial origin. It has been demonstrated that various bacteria liberate naked plasmids and

chromosomal DNA to the surroundings during spore formation, during competence development in cells that are very much alive and when cells are dying (Crabb *et al*., 1977; Lorenz *et al*., 1991; Lorenz & Wackernagel, 1994). Vesicles originating from the cell membrane, which contain both chromosomal and plasmid DNA, have been found in 14 gram-negative species of bacteria. These "blisters" were able to transfer DNA to other bacteria in the environment (Dorward & Garon, 1990).

Many extraction methods exist to analyse for DNA in the environment (see, for example, Torsvik & Goksøyr, 1978; Steffan *et al*., 1988). All told, larger amounts of DNA are extracted directly from the soil than can be achieved by extraction from the cells in the soil (Steffan *et al*., 1988), thus serving as direct evidence of the occurrence of free DNA. *Investigations also exist which show that naked DNA molecules in soil originate from micro-organisms which are no longer present in the habitat* (Spring *et al*., 1992), *yet another indication that phenotypically and genetically dead are two quite different things in an ecological context.*

In the few studies that have been undertaken (Paul & David, 1989; Lorenz & Wackernagel, 1994), the genotype, whether the organism concerned is a wild type or has been genetically engineered, has not had any influence on the liberation of DNA by the bacterial population. Liberation of DNA must be looked upon as a physiological process, but its extent may be significantly affected by abiotic (e.g. ionic strength, pH, temperature) and biological factors. Among the last mentioned are bacteriophages, which are both far more widespread and have a broader host spectrum than previously assumed (Børsheim, 1993), and protozoans (Turk *et al*., 1992).

Bacteria in natural habitats are often starved, and many species are found in a living but not cultivable form (Kaprelyants *et al*., 1993). Such cells preserve their genetic information. This was shown in non-cultivable *E. coli*, where a recombinant plasmid was stable after 28 days in an artificial seawater microcosm (Byrd *et al*., 1992). This illustrates that *genetically modified, living, but non-cultivable bacteria can be sources of biologically active naked DNA in Nature when, often after long periods, the integrity of the cells is lost* (Lorenz & Wackernagel, 1994).

6.2 Protection of naked DNA in Nature

Particles found in soil and sediment, such as quartz, feldspar and clay minerals, as well as those suspended in naturally occurring water, have the ability to bind both organic and inorganic material. When DNA is bound to some of these types of particles, it is protected from being broken down and must therefore be looked upon as a source for the transfer of genetic information (Lorenz & Wackernagel, 1994; Nielsen *et al*., 1998). Parameters which influence the speed and scale of DNA binding are the *type of mineral*, *the valency and concentration of cations and pH in the bulk phase*, whereas the temperature, DNA conformation and size of molecules seem to have little or no effect (Lorenz & Wackernagel, 1994). Clays have up to 700 times higher binding activity than quartz sand. Adsorption of DNA to minerals takes place very rapidly, and when the complexes are first formed they are very stable. *Increased concentrations of multivalent cations and low pH will increase the amount of adsorbed DNA* (Romanowski *et al*., 1991, 1993; Khanna & Stozky, 1992; Lorenz & Wackernagel, 1992).

Another important phenomenon, documented in many studies (reviewed by Lorenz & Wackernagel, 1994 and Nielsen *et al*., 1998), is that adsorbed DNA is much more resistant to enzymatic breakdown than DNA dissolved in a liquid phase. 100 to 1000 times more DNase 1 or *Serratia marcescens* nuclease is required to break down adsorbed DNA than the same amount of DNA in solution (Romanowski *et al*., 1991, 1993; Ahrenholz *et al*., 1994).

6.3 Degradation of DNA in Nature

A rule of thumb would be that plasmid DNA in waste water will be completely broken down (converted from supercoiled helix to open circles or linear forms) within minutes. In fresh water and sea water, the same process takes hours, whereas DNA can persist intact for weeks, even months or years, in soil (Romanowski *et al*., 1992, 1993) and marine sediments (Novitzky, 1986). *Then, in addition, in all these case, there is the unanswered question of whether open circles and linear plasmid and chromosomal DNA can have undesirable biological effects, too, since these forms are equally well taken up by competent cells and their genetic information can be activated by cellular processes* (Lorenz & Wackernagel, 1994; Nielsen *et al*., 1998).

Naked RNA can be identified for up to 2 days in unfiltered and 28 days in filter-sterilised sea water under experimental conditions (Tsai *et al*., 1995), and has also proved to have an amazingly long survival time in soil (Greaves & Wilson, 1970).

6.4 Uptake of nucleic acids in the mammalian organism

In many biological systems, it has been demonstrated that mammalian cells can take up foreign DNA in a manner that permits biological activity. This is, of course, precisely the basis for transfections in cell cultures, genetic modifications of plants and animals, gene therapy and DNA vaccination. However, are the epithelial surfaces in the gastrointestinal or respiratory tracts of the mammal impervious barriers to the uptake of introduced foreign DNA, or can such DNA penetrate into the organism from the extensive epithelial surfaces in the body?

The fate of nucleic acids in the gastrointestinal tract was studied in ruminants and rats in the 1970's and 1980's (Maturin & Curtiss, 1977; McAllan, 1982). The limited sensitivity of the methods available at that time meant that lack of discoveries could not exclude that biologically active DNA could both be taken up from the intestinal tract of the individual, and be dispersed to the surroundings in the faeces.

These questions have recently been re-evaluated using new, much more sensitive methods (Schubbert *et al*., 1994). Mice were pipette-fed with circular or linear double-stranded M13 bacteriophage DNA, or this was added to the feed pellets. Sensitive hybridisation methods and PCR were then used to identify M13 sequences in the faeces and blood.

The results showed that 2-4% of the introduced M13 DNA could be identified in the faeces and 0.01-0.1% in the blood, where the DNA was found in both the serum and the cell fraction. Separate

fragments measuring up to 1692 bp out of the 7250 bp total size of the M13 genome were found up to 7 hours after uptake. No difference was found between circular and linear DNA.

In more recent work, the same research group demonstrated that, under some circum-stances, ingested DNA may be taken up from the intestine of mice, inserted into chromo-somes and verti-cally transmitted to offspring (Doerfler *et al*., 1997; 1998; Schubbert *et al*., 1997).

The authors assume that other types of DNA could behave in the same manner, but they add that this must be investigated experimentally. Their observations raise several challenging questions.

To what extent can DNA which is taken up from the intestine be internalised by cells in various organ systems? Can foreign DNA in the blood stream of a pregnant female pass across the placenta and enter the foetus (Doerfler et al., 1998)? Can foreign DNA which is taken up from the intestine contribute to mutagenesis and oncogenesis? Can DNA which is released by way of the faeces be taken up by other organisms and can this have biological consequences? To what extent do the answers depend upon the DNA's sequence, structure and complex formation with proteins in the host organism, and pollution in the environment, etc.?

The genomes of the polyoma viruses (SV-40, BK virus, mouse polyoma, etc.) are small (ca. 5 kbp), circular, double-stranded DNA molecules which are able to function as expression vectors in mam-malian cell cultures. Transfection of cell cultures with naked, genomic polyoma virus DNA results in infection with production of virus particles. In a series of viral infection trials carried out at the Department of Virology in the University of Tromsø, one of the controls was naked genomic virus DNA injected intravenously into rabbits and mice. Based on what was known from the literature, and so-called conventional wisdom, it was assumed that DNA under such circumstances would be rapidly broken down by nucleases and, in practice, be devoid of biological activity. It was therefore most surprising, as well as being a lesson to us, that both viral genetic expression and full, produc-tive viral infection were indeed initiated in the animals (Rekvig *et al*., 1992; Fredriksen, 1993; Fredriksen *et al*., 1994).

That nucleic acids are taken up and have biological activity is obviously not a general phenomenon. Throughout the history of evolution, animals and people have been receiving foreign DNA from other animals and plants through uptake of nutrients and breathing of air. The problem is just, yet again, that we know that in the case of a few, perhaps rare, combinations of nucleic acids and circumstances, nucleic acids will be able to be taken up from the mucous membranes. However, we have no knowledge of the sequences, structures or environmental factors which can contribute to such stability. Nor can we therefore, at the present time, predict what type of DNA will avoid rapid breakdown in the organism and which environmental factors may contribute to this.

6.5Nucleic acid receptors

Oligo- and polynucleotides cannot diffuse through the lipid membranes of living cells. In some eucaryotic cells, it has been shown that nucleic acids can be taken up by endocytosis which is mediated by nucleic acid-specific receptors (Loke *et al*., 1989; Yakubov *et al*., 1989; Vlassov *et al*., 1994), and similar mechanisms may be active in bacteria, too (Dreiseikelmann, 1994; Lorenz &

Wackernagel, 1994). Following uptake, the nucleotides find a way of escaping from the endosomes to eucaryotic cells and reach nucleic acids that are located in both the cytoplasm and the nucleus (Vlassov *et al*., 1994). Bacteria remove the foreign DNA that was taken up using restriction enzymes which distinguish between their own and foreign DNA, but this mechanism can clearly also fail under certain circumstances (for a recent review, see Nielsen *et al*., 1998).

The biological and evolutionary importance of these mechanisms is not known and we have no knowledge about the difference between nucleic acids that are taken up in biologically active form and those that are broken down. Nor do we know whether environmental conditions can increase or reduce the expression of the nucleic acid receptors, or whether this can affect the uptake and further handling of nucleic acids in these cells.

7. Genomic re-arrangements and illegitimate recombinations

Major genomic re-arrangements, such as duplications, delesions, translocations or insertions (integrations), are important for evolution. Duplications give, for example, additional copies of genes, and these can accumulate mutations, thereby offering opportunities for further evolution (Anderson & Roth, 1977). Translocations and delesions can fuse genes, thus creating proteins with new combinations of functional domains, or change the surroundings of one gene thereby helping it to be influenced by new regulatory mechanisms. *Insertions of foreign DNA into a genome are important steps in horizontal gene transfer and help to overcome the need for repeated evolution of similar functions in different organisms (Arber, 1984). In the context of evolution, they may be looked upon as some of the positive effects of genomic re-arrangements.*

However, genomic re-arrangements may also have serious damaging effects when they occur at the wrong place, at the wrong time, or on an abnormal scale. Re-arrangements may be the cause of cell growth aberrations, and the degree of re-arrangements often increases during the development of malignant growths (Croce, 1987). They can cause the death of foetuses and developmental defects, metabolic illnesses and hereditary disorders such as Duchenne & Becker's muscular dystrophy (Bakker *et al*., 1987).

Such genomic re-arrangements may be the result of ***legitimate*** recombination which takes place between long, homologous sequences (Anderson & Roth, 1977, 1981), or what are referred to as site-specific recombination which is responsible for movements of specialised elements and genomic regions (e.g. transposons, mobile elements, etc.; Berg & Howe, 1989). However, they can also arise as a consequence of ***illegitimate*** recombinations between sequences with little or no homology (Anderson, 1987; Ehrlich, 1989; Ehrlich *et al*., 1993).

Illegitimate recombination is important because it is not confined to duplications or special, relatively rare sequences. Illegitimate recombination can therefore take place anywhere within a genome. Such recombination is probably universal since it has been found in every organism that has been searched for it, and it probably takes place far more frequently than has been envisaged (Schrempf, 1985; Ehrlich *et al*., 1993; Clegg *et al*., 1997; Gorbunova & Levy, 1997; Kusano *et al*., 1997; Dellaire & Chartrand, 1998; Zuchman-Rossi *et al*., 1998).

The general mechanisms for initiating illegitimate recombination are not well known, but there are probably several of them. Deducing the mechanisms behind a proven, naturally occurring, illegitimate recombination is very difficult because the sequence of the primary genomes is usually unknown and the re-arranged genome is first recognised after many generations. This gives time for secondary re-arrangements to have occurred, a feature often seen in studies of gene amplifications (Smith *et al*., 1990). To identify the various factors that affect illegitimate combination and determine their significance it is therefore necessary to establish model systems. So far, such model systems have mostly been established in micro-organisms, but the aim is that these will provide concepts to explain phenomena in any type of cell (Ehrlich *et al*., 1993). In spite of that, results are

now accumulating that strongly indicate that illegitimate recombination is a major driving force in the evolution of plants, animals and micro-organisms, as well as a major cause of disease (Clegg *et al*., 1997; Gorbunova & Levy, 1997; Kusano *et al*., 1997; Dellaire & Chartrand, 1998; Zuchman-Rossi *et al*., 1998). In transgenic mice (Dellaire & Chartrand, 1998) and in plants (Gorbunova & Levy, 1997), illegitimate recombination leads to the random and unpredictable integration of trans-genes in the recipient chromosomes.

It is perfectly clear that a great deal remains to be learnt before we can claim more than a very rudimentary understanding of the phenomenon of illegitimate recombination, and still more before we can hope to control it (Ehrlich et al., 1993). Genomic re-arrangements are often found to modify, in undesirable places, gene constructs that are meant to be for biotechnological use (Ehrlich et al. 1986, 1993; Gorbunova & Levy, 1997). This should perhaps, itself, give grounds for warning signals about what may happen if such processes take place after GM constructs have established themselves in an ecosystem.

8. Horizontal transfer of nucleic acids and genetic informtion

For any given gene construct or GMO which is released, or escapes, to the environment, the present state of our knowledge does not allow a pre-assessment of either the probability for, or the consequences of, horizontal gene transfer. Hence, according to the definition of risk (see Chapter 1), risk assessments are impossible at the moment, Only extensive research on the mechanisms of horizontal gene transfer and ecosystem interconnections can change this situation.

Horizontal (lateral) gene transfer is defined as non-sexual transfer of genetic information between genomes (Kidwell, 1993). The expression is generally used about transfer between core genomes in different species, but can also be applied to genetic transfer between different organs in the same or different species. Transfer with the aid of parasitic species or symbionts to host species can also be included (Timmis & Scott, 1984).

Horizontal transfer is thus distinct from the ordinary form of gene transfer which takes place *vertically* from parent to offspring. There is now good evidence that *horizontal transfer takes place for both genomic (usually non-mobile) sequences and sequences derived from transposable genetic elements or mobile introns*. Documented cases exist of genomic sequences being transferred from eucaryotes to procaryotes, from procaryotes to eucaryotes, between procaryotes and between eucaryotes (reviews in Heinemann, 1991; Kidwell, 1993; Harding 1996; Wöstemayer *et al*,. 1997 and Nielsen *et al*., 1998).

The possibility that genetic information could move between distantly related species was an idea which met a great deal of opposition in traditional biological schools when it was first put forward a couple of decades ago (Syvänen, 1994). The opposition is understandable enough, because this concept, viewed superficially, conflicts with both explanatory models based on phylogenetic trees in taxonomy and the important role of reproductive isolation as a mechanism for species formation. However, horizontal gene transfer is now gaining more and more support. Not only are there dozens of examples of probable horizontal transfer, but the molecular mechanisms which may contribute to such transfer are continually being observed, both physical means of transfer for DNA between cells and recombination mechanisms which can lead to the gene transfer becoming permanent. Horizontal transfer of genes is now an indisputable fact, and the most important question that remains is whether such transfer takes place at a speed that significantly affects evolution.

The debate has mainly concerned horizontal transfer of entire genes, but for *E. coli, Streptococcus* and *Neisseria* species it has been shown that far shorter elements are stably transferred and can give rise to mosaic genes (review in Lorenz & Wackernagel, 1994). There are strong indications that this takes place in eucaryotic organisms, too, exemplified by cytochrome c in plants and betaglobines in mammals (Syvänen, 1994). *Theoretically, shorter DNA sequences will, without our knowledge, be able to contain control elements for expression of genes (e.g. promotors or enhancers) which can change the amounts of some gene products in the recipient, perhaps with substantial biological consequences*.

A general evolutionary theory which incorporates horizontal transfer of genes astride taxonomic boundaries seems to be able to give a satisfactory answer to the important question: *Why is the molecular biology of all living organisms so uniform?* Despite species formation, biology has maintained a uniformity which even permits transgenic animals to be constructed in the laboratory.

Many evolutionists still believe that an evolutionary theory that incorporates horizontal transfer conflicts with the useful concepts, "phylogenetic trees" and "reproductive isolation". It is far simpler for molecular and cellular biologists to accept it. However, the widespread horizontal transfers that have taken place for sub-populations of *E. coli* and *S. typhimurium* have not prevented these bacteria from being capable of being placed in phylogenetic, tree-like evolutionary models (Syvänen, 1994). Some microbiologists who studied plasmids assumed at an early stage that cross-species gene transfer took place, and used this as the basis for arguing against the feasibility of a meaningful phylogenetic classification of bacteria. Possibly, they did not go too far (for review, see Nielsen *et al*., 1998).

We thus know that there are limitations as to <u>what</u> kind of DNA can be transferred, but we do not know the kinds of mechanisms which sort DNA for transfer and are therefore unable to pre-assess <u>whether</u> a plasmid or another genetic construct which we make use of will be transferred horizontally, <u>when</u> it will transferred and <u>where</u> it will end up. Furthermore, as Nielsen et al. (1998) wrote: "Transfer frequencies should not be confounded with the likelihood of environmental implications, since the frequency of horizontal gene transfer is probably only marginally important compared with the selective force acting on the outcome".

For any given gene construct or GMO which is released, or escapes, to the environment, the present state of our knowledge does not allow a pre-assessment of either the probability for, or the consequences of, horizontal gene transfer. Hence, according to the definition of risk (see Chapter 1), risk assessments are impossible at the moment, Only extensive research on the mechanisms of horizontal gene transfer and ecosystem interconnections can change this situation.

Evolution favours those organisms which have a suitable balance between genetic variation and genetic stability. However, we do not know how such a balance is established and maintained, and consequently neither whether, nor how, it can be upset.

8.1 Barriers to horizontal transfer

For horizontal transfer to take place, genetic material has to overcome at least two types of hypothetical barrier (Heinemann, 1991), an *<u>introduction barrier</u>* and an *<u>establishment barrier</u>*. These barriers ought to make contact between genetic donors and recipients difficult, degrade genetic material, exclude foreign material from replicational and/or segregational processes, and prevent the expression of genes which are required for inheriting transferred molecules. *It is clear that the introduction barriers are often broken and that a network for genetic exchange between organisms exists.*

Many observations and experiments indicate that introduction barriers are often broken so that DNA wanders between phylogenetically remote species (Heinemann & Sprague, 1989; review in Heine-

mann, 1991). Many bacteria may be naturally competent for being exposed to transformation with DNA from any source whatsoever (Heinemann, 1991). Conjugational transfer of DNA does not only take place within species, but also across species boundaries, and even kingdoms. Agrobacteria may, for example, transfer DNA to their plant hosts, and effective conjugation can take place between *E. coli* and several yeast species (Stachel & Zambryski, 1989). This, in turn, indicates that *establishment barriers are very effective and that they are necessary for species to be able to remain distinct in a world of genetic promiscuity* (Heinemann, 1991).

The problem is that we know that establishment barriers, too, may be broken, but we do not know the mechanism and can therefore not guard against such highly undesirable occurrences. We undertake modifications and mutations which are intended to make nucleic acids more effective in use. Examples have been published where small changes in a DNA sequence can change the host spectrum for a transferable genetic element (Kipling & Kearsey, 1990). Do we undertake, without being aware of it, such changes with our genetic constructs and modifications? Are the barriers capable of being influenced by the amounts of naked DNA, and how much DNA is required in a given situation to break down an ecological barrier? Finally, it is also in this context important to remember that the plasmids used for immunisation and gene therapy are both procaryotic and eucaryotic shuttle vectors. Consequently, if they escape into Nature they can multiply in and disperse with representatives of both kingdoms.

8.2 General mechanisms for horizontal gene transfer

The occurrence of, and mechanisms for, horizontal (lateral) transfer of genes have been remarkably little studied, especially in eucaryotic cells and organisms. However, there are some brief and useful reviews of the topic (Heinemann, 1991; Landman, 1991; Bogosian & Kane, 1991; Powers *et al*., 1991, Thakur *et al*., 1991; Kidwell, 1993; Lambowitz & Belfort, 1993; Dreiseikelmann, 1994; Lorenz & Wackernagel, 1994; Capy *et al*., 1994; Harding 1996; Wöstemayer *et al*,. 1997 and Nielsen *et al*., 1998).

It is usual to distinguish between the following general mechanisms:

i) *Transduction* Foreign genetic material that has been included in a virus genome can be transferred to a new cell which is infected by the virus, a bacteriophage in bacteria (review in Dreiseikelmann, 1994) or, for example, retroviruses in mammalian cells.

ii) *Conjugation* Genetic material on plasmids which also steer the transfer process itself. Requires close physical contact between two bacteria cells. Occurs more often than assumed between bacteria which are only distantly, or not at all, related. Conjugation can take place between all gram-negative species, and also from gram-negative to gram-positive species. Both *E. coli* and the plant pathogene *Agrobact-erium tumefaciens* can transfer DNA to eucaryotic species (review in Heinemann, 1991). The conjugation phenomenon is partly responsible for the development of antibiotic resistance in bacteria. Resistance determinants are often found on plasmids which direct conjugation between bacteria and often group themselves with other resistance determinants. The spread of antibiotic resistance factors through populations of pathogenic and non-pathogenic bacteria demonstrates the potential for horizontal transfer of genes across large geographical distances in a short time.

iii) *Transformation*, often called *transfection* when it concerns mammalian cells. Free DNA is taken up in cells. Some bacteria do this under many conditions; most can probably do it under certain conditions. Single-celled eucaryotes often easily allow themselves to be transformed. Cells from multi-cellular vertebrate organisms, as shown by DNA vaccination trials (see section 5.1.1), more effectively take up naked DNA than previously thought. DNA will often be in complexes with proteins and can be taken up in cells by receptors directed towards these proteins.

iv) *Transpositioning* Genetic information is moved to other parts of the genome, to plasmids or to other genomes with the help of what are called *transposable* or *mobile elements*. Since such elements were discovered in the 1940's by the subsequent Nobel prize winner Barbara McClintock, various forms have been discovered in practically all the species investigated in the three kingdoms (archebacteria, procary-otes and eucaryotes). Several examples of a combination of conjugation and trans-positioning are known in bacteria. Plasmids can collect together and deliver trans-posable elements to new host cells by either conjugation or transformation (Heinemann, 1991).

8.3 Proving horizontal transfer of genes

Enforced transfer of genes between species from different kingdoms has become a routine procedure in molecular biological and biomedical laboratories during the last 20 years. The general success of these artificial genetic transformations, over broad taxonomic areas, has renewed the interest for earlier speculations and circumstantial indications regarding the frequency and significance of *natural* horizontal transfer of genes.

It is difficult, frequently impossible, to conclusively prove that horizontal transfer, in contrast to some other genetic occurrence or mechanism, has taken place. However, improved means of retrospectively proving horizontal transfer have recently been developed. Polymerose chain reactions (PCR), along with rapid and inexpensive techniques for DNA sequencing, reveal genetic variations which are inconsistent with vertical transfer of genes (Kolstø & Prydz, 1994; Nielsen *et al*., 1998).

Generally, analysis of sequence data give phylogenetic trees which are compatible with strictly vertical gene transfer. *Lateral* transfer can be *suspected* when anomalies are shown to exist in trees based on sequence data, but other explanations must be considered and the following criteria should be used (Smith *et al*., 1992):

1. Sequence data should be available for a number of evolutionary distant organisms.

2. Apart from the divergent taxon which is investigated, the other taxons should show sequence changes that are compatible with conventional phylogenies.

3. Trees must be well "rooted" in sequence data from a more distantly related species if the classification is not to be debatable.

4. Concurrent results from several methods of phylogenetic analysis strengthen the suspicion of horizontal transfer.

5. It is an advantage if conventional gene transfer can be proved for a separate set of sequence data from the same phylogenetic group.

The simplest situation is obviously that a gene is found in one species, but is absent from closely related species (Kidwell, 1993).

Proof exists for natural horizontal transfer of genes which concern genomic sequences, i.e. those which are not normally mobile, and also for sequences originating from transposable or mobile genetic elements.

8.4 Horizontal transfer of genes in procaryotic micro-organisms

8.4.1 Dogmas and lack of knowledge

If new genetic information, for instance in the form of recombinant DNA, from released or escaped GMOs is introduced to an ecosystem, the chances of permanent impacts on the equalibria in the system will be greatly enhanced if the nucleic acids are taken up by species that are naturally present in, and better adapted to, the special habitats than the introduced GMOs.

One reason for the lack of knowledge and research effort in this field is the apparently reassuring data published following studies of the possibilities for dispersal in the environ-ment of strain K-12 of *E. coli* and for conjugational transfer of the most frequently used basis plasmid of *E. coli*, pBR322, to other bacteria (review in Bogosian & Kane, 1991). Small-scale trials implied that K-12 had a maximum survival time of 15 days in water, 10 days in sewage and 6 days in the intestinal system of man or mice. These were looked upon as such short survival times that they did not entail health or ecological risks, an opinion we now know to be wrong. Conjugation-based transfer of plasmids to naturally occurring bacteria was only proved under very special conditions.

Both these conclusions had to be expected in view of the phenotypical characteristics of the bacterial strain and the plasmid, but they have subsequently found general application for uncritical dismissal of environmental risks associated with recombinant nucleic acids and organisms.

Such generalisation is completely non-scientific, because the conclusions were drawn on the basis of small-scale trials with poorly representative bacteria and plasmids under barely authentic conditions. Short-term experimental strategies were used on a phenomenon that has long-term perspectives. The detection methods had poor sensitivity considering the time span of the trials. Only a single mecha-nism for DNA transfer to new host bacteria was investigated, namely conjugation-based plasmid transfer. There was no awareness that genetic death, i.e. biological inactivity when nucleic acids are broken down, takes considerably longer than phenotypical death (loss of the life functions of the bacteria). It was not known that the properties of the DNA sequences which were inserted in the recombinant plasmids can substantially affect both survival and chances of transfer. It was not

known that partially broken down plasmids can be taken up by both procaryotic and eucaryotic (e.g. human) cells and have biological effects.

The relatively uncritical acceptance of these early trials brings little honour to researchers. They belong to a profession whose task is to be critical to established truths, not to establish dogmas themselves which serve their own purposes or those of their clients.

The *E. coli* K-12 strain and derivatives of pBR322 are still two of the most important tools in modern genetic engineering production systems. Such production includes large-scale fermenting embracing some hundreds to thousands of litres of bacterial and cell cultures. This means that up to 10^{17}, or more, recombinant *E. coli* cells can be released into the environment following an accident at a manufacturing firm. No studies have so far tackled the complicated, but vital, question of the possible ecological significance if any transfer of genes whatsoever occurs, even with very low frequency, in such a situation (Bogosian & Kane, 1991).

8.4.2 Relative importance of DNA uptake mechanisms

Horizontal transfer of accessory DNA elements is assumed to be one of the most important factors in the adaptation to the environment of the microbial community and for the evolution of new metabolic pathways within the community. Plasmids are elements which are assumed to constitute important parts of the horizontal gene stream. Plasmid genes often code for products that give selective advantages in stressed or hostile environments (Trevors *et al*., 1987), and plasmids are able to undertake autonomous replication and promote their own transfer. With the help of *simple conjugation*, many trials have produced bacteria which have new biodegradable properties (see, for example, Latorre *et al*., 1984, Smets *et al*., 1993).

Co-cultivation of micro-organisms which have different, unique degradation genes on plasmids have led to genetic re-arrangement which, in turn, has resulted in one micro-organism having acquired catabolic functions which were not found in, or could not have been derived directly from, any of the parent strains (see, for example, Kellog *et al*., 1981).

Many questions have to be answered before a complete picture can be painted of the fate of conjugating plasmids in the microbial community. Smets *et al*. (1993) showed that the rate of conjugation in densely populated microbial communities may be large enough to maintain a plasmid-coded gene when horizontal dispersal takes place. However, the authors themselves point out that their dispersal frequencies represent minimum values because the experiments were carried out in suspension cultures, whereas the microbial biomass in many natural systems occurs as aggregates or on surfaces, and this can considerably affect the frequency.

Transduction is another potentially important mechanism for gene dispersal in the environment. The possibility of transferring both chromosomal and plasmid-localised genes between bacterial populations on plant surfaces and in aquatic environments using this mechanism has been demonstrated in many studies (Ogunseitan *et al*., 1992; Zhou *et al*., 1993; Dreisekelmann, 1994), but has not been attributed any importance in a risk context.

Biotechnological solutions to problems in agriculture often entail the release and dispersal of GMOs to surfaces on crops and other plants. Recent studies have shown not only genetic exchange by transduction between bacterial populations on the same plant, but also transfer to other plants when this is made possible by closely-spaced planting (Kidambi *et al*., 1994).

The efficiency of horizontal gene transfer between microbes is dramatically illustrated by the occurrence of antibiotic-resistant genes, even in apparently noncontaminated biotopes and ecosystems (see Davies, 1994; Kruse, 1994; Andersen & Sandaa, 1994; Kruse & Sørum, 1994; Kruse & Jansson, 1997). Nikolich *et al*. (1994) demonstrated naturally occurring transfer of a tetracycline-resistant gene between intestinal bacteria (*Bacteriodes* spp. and *Prevotella* spp.) from domestic livestock (pigs, cattle, sheep) and man. There has been speculation for a long time that this could occur, but this is the first unambiguous proof. Gene transfer can occur both plasmid-bound and with the help of conjugative chromosomal transposons. These bacteria constitute a far greater proportion of the intestinal flora than *E. coli* and the facultative gram-positive cocci that are often focused upon. Likewise, Kruse & Sørum (1994), in an elegantly planned and implemented model trial, demonstrated the transfer of R plasmids astride a number of conventionally recognised borders on both bacterial-species and eco-niche levels.

Transformation means that free DNA is directly taken up by bacteria, and the term *natural genetic transformation* is used to differentiate uptake occurring under natural conditions from artificial laboratory procedures. Natural genetic transformation can be looked upon as the most widespread transfer mechanism, since the other known mechanisms are steered by genes located on plasmids or transposons (*conjugation*), or on bacteriophages (*transduction*).

The special terms that have to be fulfilled for the implementation of these three transfer mechanisms imply different probabilities of them occurring under natural conditions.

Conjugation places the strictest demands. The donor cell must contain conjugative elements (plasmid or transposon). The donor and recipient cells must establish physical contact that is sufficiently stable to permit the transfer of DNA. Both cells must be metabolically active to permit DNA synthesis and other essential activities (Ippen-Ihler, 1989; Clerc & Simonet, 1998).

Transduction requires a metabolically active donor cell which is producing transducing bacteriophage particles. The recipient cell may be separate from the donor in space and time, because the genetic information is preserved in the bacteriophages which are resistant to any physical and chemical effects and can survive longer under natural conditions, especially if they are adsorbed to clay and other particulate minerals (Stotzky, 1989). However, an absolute demand is that a relevant recipient must be receptive to the same bacteriophage, whereas metabolic activity is unnecessary (Kokjohn, 1989).

Transformation does not even require living donor cells, because liberation of chromosomal or plasmid DNA at death, and cell lysis, are sufficient to maintain available DNA to transfer genes. How long DNA can "survive", and its dispersal in the surroundings, determine how far the donor and recipient can be separated in space and time (Leff *et al*., 1992). The recipient must be to some extent physiologically active to be able to take up DNA. Close genetic relationship is unnecessary, especially for naturally occurring transformation with plasmid DNA (Lorenz & Wackernagel, 1994).

Natural genetic transformation therefore has several characteristics which mean that it can take place within populations and biological communities that undergo extreme environmental changes and dramatic fluctuations in population dynamics (Lorenz & Wackernagel, 1994; Nielsen *et al*., 1998).

However, the fact that transformation is probably the most general of the three obviously does not preclude the possibility that natural links and interactions exist between the three transfer mechanisms. A genetic element that enters an ecosystem by one of these mechanisms can thereafter be carried further by the others.

It has been shown that extracellular DNA is present in natural environments and that such DNA can be taken up in bacteria. Simulation trials have demonstrated that many bacteria can achieve competence for DNA uptake under conditions which can arise in natural habitats. These observations are compatible with bacterial gene transfer performed by free, naked DNA taking place on a significant scale (Lorenz & Wackernagel, 1994).

The various methods employed to make bacteria competent for genetic transformation under laboratory conditions can be divided into the following main groups (Mercenier & Chassy, 1988): i) treatment with solutions of calcium chloride or chlorides of other elements, including magnesium, barium, rubidium, strontium and mixtures of such heavy metals; ii) treatment with EDTA or other chelating substances; iii) treatment with enzymes (e.g. muraminidases or proteases); iv) fusion of cells with DNA, other cells, or DNA packed into liposomes; v) freezing and thawing of cells; vi) exposure of cells to electric fields; vii) bombardment of cells with small particles which transport DNA into cytoplasm (biolistic transformation).

It is not difficult to envisage that bacteria in the ecosystems can be exposed to conditions which coincide with the laboratory procedures listed above. It is also easy to imagine that pollution with heavy metals, etc. may have a significant effect on these conditions.

8.4.3 Competence

In this context, *competence* is defined as the ability to take up free DNA from the ambient medium. Development of competence requires the expression of specific genes whose proteinic products supply essential functions. For some bacteria, microcosmic conditions have been defined which lead to the development of competence and the uptake of DNA, but this does not apply to many species. The differences, in this context, between strains of the same species of bacteria are striking, and frightening (Lorenz & Wackernagel, 1994; Nielsen *et al*., 1998). A list of the species of bacteria for which it is known that natural competence occurs would at present number more than 40 isolated from terrestrial and aquatic habitats, as well as a number of isolates of human and animal pathogenic species. Nevertheless, it is strongly emphasised that this concerns species in which the phenomenon is *known*. It must be expected that natural competence can occur in a great many species which have not been investigated for it, where the correct strains have not been investigated, or where appropriate conditions for competence development are not achieved in the laboratory or the microcosm. It is also important to remember that competence is temporary in most species (Lorenz & Wackernagel, 1994). *Neisseria gonorrhoeae* is at present the only species known whose natural competence seems to be constitutive (Sparling, 1966).

It is of interest to note that some bacteria develop competence more easily in simple than in complex media (Page & Sadoff, 1976; Dubnau, 1991). A reduction in capsule thickness in a *phosphate-rich medium* (cf. phosphate emissions) may enhance the competence (Page, 1985). Relative iron deficiency may also lead to increased competence for some bacteria. Divalent calcium and magnesium ions are important for competence in several bacteria. *Calcium can be replaced by strontium, but magnesium cannot*. The calcium concentration for optimal competence development may, for example, range from 0.5 to 1 mM, and such values can often be measured in soil, sediment and water (Page & Doran, 1981; Lorenz & Wackernagel, 1994).

8.4.4 DNA binding, internalisation and transport

It may be that DNA binding takes place to nucleic acid receptors on the surfaces of many bacteria, but experimental proof of this only seems to exist for *Haemophilus influenzae* (Goodgal, 1982). The actual internalisation process for DNA has been studied for a few species (Smith *et al*., 1982; Mejean & Claverys, 1988, 1993; Dubnau, 1991). What seems clear is that both the actual competence and the effectiveness of the uptake can be drastically influenced by environmental conditions, which may vary significantly in natural habitats, and from one habitat to another (Garcia *et al*., 1978; Romanowski *et al*., 1993). Transport into the cell takes place with the assistance of special vesicles called *transformasomes*, and the models from both conjugation and transformation include a protein canal that spans the entire bacterial wall. *However, there are great differences from bacteria to bacteria regarding the kind of DNA that is transported most effectively. Very good review articles have recently dealt with this topic (Dreisekelmann, 1994; Lorenz & Wackernagel, 1994; Nielsen et al., 1998). The only possible conclusion is that it is impossible to draw any general conclusions.*

8.4.5 Genetic transformation under natural conditions

Competent bacteria cells have to exist in the environment (the habitat), and a number of specific conditions also have to be satisfied: i) the bacteria must come into contact with DNA; ii) the DNA must have a minimum length; iii) specific cations must be present in optimal amounts; iv) the DNA must follow an internalisation route which gives biological meaning.

These rules apply for chromosomal DNA; investigations of free plasmid DNA have not been reported. Model studies, however, show that plasmid DNA is liberated from bacteria together with chromosomal DNA (Lorenz *et al*., 1991). Such liberated plasmids maintain their transforming activity in soil (Romanowski *et al*., 1992, 1993).

Transformation in a microcosm

A number of experimental studies have been performed to investigate genetic transformation in other habitats than inside macro-organisms; for instance, in soil, sediment and water. The experiments have been done in a microcosmos set-up using samples taken from natural habitats. Most of the studies concern the ability for transformation in bacterial strains which are introduced into a microcosm. They relate to the development of competence in the strains, and their ability to take up DNA and to express marker genes which they acquire in a microcosm. *The obvious, main conclusion*

to be drawn from these studies, which have taken place over many years, is that in no instances are there any specific model organisms which are representative for other naturally transformable bacteria in a given habitat (Lorenz & Wackernagel, 1994). Naturally occurring genetic transformation has been proved in all kinds of habitat, but otherwise the results are often highly contradictory. This probably only reflects that it is impossible to generalise results achieved with an ecotype of a given species of bacteria (Leff et al., 1992). However, there are studies which illustrate that recombinant strains resulting from transformation may be better ecologically adapted than any of the primary strains (Graham & Istock, 1978, 1979, 1981).

Liberated or escaped DNA is distributed between the fluid phase and solid surfaces. Such DNA can transform competent cells. On the other hand, both free DNA and DNA that is taken up by cells can be degraded by extra- and intracellular DNases and restriction enzymes. The products can then be used as nutrients (Lorenz, 1992).

Importance of the chemical environment

Disturbingly little has been done in this important field where it is obviously impossible to generalise because different bacteria have different requirements and where *both the natural and the polluted environments can vary enormously over time and from place to place.* Nevertheless, there are some studies of microcosms which show that bacteria *do* become transformed under natural conditions and that the effectiveness of this can be influenced by biotic as well as abiotic factors (Lorenz & Wackernagel, 1991, 1992; Paul *et al*., 1991; Frischer *et al*., 1993; review in Lorenz & Wackernagel, 1994).

Transformation on solid surfaces

It has been shown that many bacteria are transformed by DNA bound to sand and clay particles (review in Lorenz & Wackernagel, 1994). For some species, the transformation is more, for others less, effective than with corresponding DNA concentration in solution.

Complexity of ecosystems

Most microcosmos studies have been concerned with variations in bulk soil (Nielsen *et al*., 1998), but real ecosystems are very complex, varying in soil composition, chemical pollution and organisms. Horizontal gene transfer is also likely to take place within the digestive systems of protozoa, nematodes, insect larvae, earthworms and other soil-inhabiting macro-organisms (Adamo & Gealt, 1996; Daane *et al*., 1997; Schlimme *et al*., 1997).

The fate of internalised DNA

Since a competent cell in the environment will be capable of taking up DNA which derives from many different organisms that are present, have been present, or have had their DNA introduced to the habitat, it is important to consider factors which influence the fate of DNA in the cell.

Studies of such factors have often brought surprising results. For instance, some bacteria will correct mismatches that arise between their own DNA and DNA which they take up, others will not. If the non-homology of an allele is increased, it will reduce the effectiveness of transformation in some and increase it in others (reviews in Lorenz & Wackernagel, 1994 and Nielsen *et al*., 1998). *It is impossible to generalise or extrapolate*.

Transfer of chromosomal DNA across species' boundaries

A number of studies prove that chromosomal genes can be transferred by transformation between species and even across higher taxonomic boundaries (Lorenz & Wackernagel, 1994).

Most of the interspecies, chromosomal gene transfer is assumed to take place between species within the same genus. Nevertheless, the variations are formidable even at that level. Within the genus Haemophilus, for example, a difference of up to 25,000 times has been recorded in how effective transformation is between related bacterial strains with the same DNA, and this difference was not related to the degree of genetic kinship (Albritton et al., 1984). Similar discrepancies between the degree of kinship and the effectiveness of transformation have also been shown for other model systems (Harford & Mergeay, 1973; DeLey, 1992). The reason(s) for this are completely unknown.

Plasmid transformation across species boundaries

The transfer of plasmids between cells from different species require that the plasmid can initiate replication in many different hosts. The plasmid is taken to the nucleus of the recipient as single-stranded fragments. Reconstitution of the circular, double-stranded plasmid DNA molecules does not require sequence homology to the recipient's DNA, but, on the contrary, recombination and repairing enzymes which are compatible. In some situations, it seems that transformation can only occur with simultaneous uptake of chromosomal and plasmid DNA (Chamier *et al*., 1993), but this is after all likely to be the natural scenario, too. In *E. coli*, transposable sequences can be shuffled back and forth between the positions where genomes and plasmids are located and be transferred to other bacteria with the help of promiscuous plasmids.

Even though special conditions are stipulated and varying restriction enzyme systems are present in different species, plasmids and transposons can be transferred between all the different parts of the procaryotic world (Lorenz & Wackernagel, 1994). For recombinant shuttle vectors, which are constructed to function in both procaryotic znd eucaryotic hosts, the picture will obviously be both more complicated and unpredictable.

Naturally occurring transformable bacteria

The very first attempt at transformation that has been described (Griffith, 1928) still illustrates ecologically important aspects. Dead, pathogenic (S-shaped) bacteria could transfer their pathogenic properties to non-pathogenic (R-shaped) bacteria following injection into mice. This illustrates the difference between phenotypic and genetic death, and that genetic life may have dramatic ecological consequences in the event of transformation or other means of genetic transfer. For many natural populations of pathogenic bacteria the potential for natural genetic transformation seems to be great. Among clinical isolates of *Streptococcus pneumoniae* and *Haemophilus influenzae*, 6 of 9 (66%) and 13 of 31 (42%), respectively, proved to be transformable (Yother *et al*., 1986; Rowji, 1989). Of 10 clinical isolates of *Heliobacter pylori*, 3 proved to be naturally competent, but with one hundred times difference in their transformation frequency (Haas *et al*., 1993).

Many habitats exist which have a high potential for gene exchange by transformation. One example is tubers which contain large quantities of rhizobacteria. These are able to develop natural competence and transport free DNA into their cytoplasm. Other examples of localities with high concentrations of bacteria which may favour transformation of free DNA, or gene transfer by cell contact, are

the intestines of both vertebrates and invertebrates, protozoans and surficial, mesophyllic and intracellular space in plants (Lorenz & Wackernagel, 1994). Possibilities for uptake of DNA in eucaryotic cells also arise here.

8.4.6 Estimates of transformation frequencies in the environment

Comparisons of the transformation frequencies in microcosm experiments which simulate natural habitats and those attained under optimised laboratory conditions reveal many minor and major differences. The differences between transformation frequencies recorded in microcosm experiments and those truly occurring in Nature may be at least as great. The reason is that *the variations in both environmental parameters like temperature, pH, supply of nutrients, minerals, particle composition, contamination, total number of micro-organisms and composition of populations, as well as the barriers to transformation cannot be simulated in the microcosm. Nonetheless, the many data amassed in recent years strongly imply that there may be high transformation frequencies in some natural habitats (Lorenz & Wackernagel, 1994; Nielsen et al., 1998).*

8.4.7 Barriers to transformation

Physiological, genetic and microcosmic studies have shown that transformation frequencies vary both within and between species (Lorenz & Wackernagel, 1994; Nielsen *et al*., 1998). Some varying factors and mechanisms exist within the cells and the environment which may limit the efficiency of transformation in natural bacterial ecosystems, and thus constitute barriers to transformation.

DNA restriction For some bacteria, and for combinations of different bacteria, transformation with chromosomal DNA seems not to be affected by the restriction-enzyme systems of the recipient bacteria (Goodgal, 1982), whereas in other cases these seem to constitute an efficient barrier (Cohan *et al*., 1991). The same variation applies for transformations with plasmid DNA (Stein, 1991; Bruns *et al*., 1992).

It is clear that more research is essential to learn the reasons why DNA restriction acts as a barrier to the transfer of genes in some cases and not in others.

Occurrence and level of competence in Nature
Great variations in the ability to transform and in the level of competence have been observed among natural isolates of species which include laboratory strains that are known to be transformable. Of 54 clinical isolates of *Acinetobacter calcoaceticus*, 2 (4%) were transformable, having frequencies of 0.3 and 1.9% of the standard laboratory strain (Bergan & Vaksvik, 1983), whereas 64% of *Pseudomonas stutzeri* and related species were transformable (Carlson *et al*., 1983). Of 771 heat-resistant bacteria isolates from desert sand, 54% were transformable (Cohan *et al*., 1991). Natural competence among marine isolates varies greatly (review in Lorenz & Wackernagel, 1994). *Natural competence is thus a completely unpredictable parameter which requires more research.*

Environmental factors

In the present context, this refers to physico-chemical factors (e.g. types and concentra-tions of ions, temperature, pH, etc.) and biological factors. Some investigations have been carried out as regards the former, but the latter have been very unjustly treated in a scientific sense. Studies in microcosms indicate that the frequencies of transformations in Nature may be high in some situations (reviews in Lorenz & Wackernagel, 1994 and Nielsen *et al*., 1998), but a number of important questions remain completely unanswered.

- *Which factors decide the transformation frequencies in a given habitat?*
- *What decides the actual concentration of transforming DNA in a given habitat?*
- *What is the difference between transforming and non-transforming DNA, and is the difference general or related to one or more specific recipients?*
- *Is access to nutrients decisive?*
- *How will different kinds of pollution affect competence development and transformation ability in different bacteria?*
- *How do the composition of, and interaction between, species within the total population influence the transformation frequency?*

The studies carried out to answer the last question show that the interactions may vary from an increase of, or a neutral effect on, to a pronounced downward regulation of the transformation frequency (Basse *et al*., 1995; reviews in Lorenz & Wackernagel, 1994 and Nielsen *et al*., 1998). To study the complicated ecological interplays, microbial habitats of increasing complexity have to be established and relevant organisms need selecting for simulation experiments.

8.4.8 Importance and extent of horizontal gene transfer between micro-organisms

The horizontal transfer of genes plays a positive role by giving genetic flexibility and adaptability to a shifting environment and also providing the basis for species formation by sexual isolation. Horizontal transfer between species offers possibilities for spreading environmentally adapted genes to more species within the same environment. Gene transfer has taken place, and presumably still does, between distantly related and non-related taxonomic groupings and also astride the taxonomic kingdoms (DeFlaun & Levy, 1989; Heinemann, 1991; Mazodier & Davies, 1991). Nucleotide sequencing and comparison of codon use and gene products show that a number of genes and chromosomes in many species are mosaics of DNA segments from different species that often have different evolutionary histories (Maynard Smith *et al*., 1991; Smith *et al*., 1992).

The extent of horizontal gene transfer may be far greater than the classical studies have succeeded in revealing (Lorenz & Wackernagel, 1994; Nielsen et al., 1998), because such studies have concentrated on demonstrating known differences in phenotypical traits over short time intervals as evidence that transfer has taken place. This only reveals the changes that are being sought, and only short-term consequences of processes are sought that may have long-term effects. Subtle quantitative differences in genetic expression may have major ecological consequences. Horizontal transfer of genetic control elements instead of, or in addition to, coding sequences may result in such differences and can also predispose for phenotypical changes which only become apparent under specific conditions. Such changes will only be found if they are deliberately sought; they include protection against bacteriophages, more efficient utilisation of nutrition, regulation of certain genetic expres-

sions, more efficient DNA repair, and resistance to a toxic product, etc. However, such phenotypical changes in single populations may have great ecological effect over time, because the balances in the ecosystems are upset. Such effects of gene transfer have been observed in model experiments. Transformation of competent H. influenzae with DNA from other Haemophilus species led, for example, to up to 55% of the recipient cells being killed, probably due to the induction of a defective prophage (Albritton et al., 1984).

8.5 Horizontal gene transfer in eucaryotes

The existence of a number of discrepancies as regards species has been recognised for many years without any ultimate explanation having been found because the available analytical methods have been too inexact to differentiate between alternative explanations. This situation has now changed with the arrival of modern sequencing methods and PCR. In the years to come, access to sequence data from many different organisms will permit more reliable estimates of the frequency of horizontal transfer of genes (Kidwell, 1993; Nielsen *et al*., 1998).

8.5.1 Genomic sequences

The horizontal transfer of genes between several distantly related procaryotes is very convincingly documented (Levy & Miller, 1989; Sprague, 1991; Mazodier & Davies, 1991; Maynard Smith *et al*., 1991; Dreiseikelmann, 1994; Syvänen, 1994; Lorenz & Wackernagel, 1994; Nielsen *et al*., 1998).

Recently, a number of previously suspected horizontal gene transfers between procaryotes and eucaryotes were re-examined in detail (Smith *et al*., 1992). Five cases were judged as probable in varying degrees, one was considered impossible, and the remainder were characterised as improbable. The probable ones concerned transfers both from procaryotes to eucaryotes, between eucaryotes and from eucaryotes to procaryotes. Eucaryotic to procaryotic transfers include transfer from plants to bacteria (Smith & Doolittle, 1989, review in Nielsen *et al*., 1998) and from mammalian virus to *E. coli* (Doolittle *et al*., 1989). *In one of these cases, there are strong indications of horizontal transfer having taken place through several stages, first from an animal to a bacterium and then continuing between several species of bacteria. This concerned the Fibronectin type III (Fn3) domain, which is common among animal proteins, but not plants and fungi, and usually not bacteria either (Bork & Doolittle, 1992).*

As mentioned above, several cases of horizontal transfer from procaryotes to eucaryotes have been documented (Smith *et al*., 1992; Nielsen *et al*., 1998), whereas there are few credible examples of such transfer of genomic, non-mobile sequences *between* eucaryotes. Those which are reported, and which are accompanied by strong circumstantial evidence, seem to involve viruses, pseudogenes or multigenic families (Kidwell, 1993). However, there is at least one well documented case, a quite "recent" transfer of retrovirus sequences from apes to a close ancestor of the domestic cat. There also exists a well-documented case of transfer between a gallinaceous bird and a rat (Kidwell, 1993).

8.5.2 Mobile elements and introns

Eucaryotic *transposable elements (TE)* can be placed in two classes depending on their transposing mechanisms (Kidwell, 1993):

Class I DNA elements known as *retroelements* or *retroides*. This class includes retro-proviruses, some DNA viruses and group II mobile introns. The elements perform transpositioning by reverse transcription of an RNA intermediary. The class can be divided into two subclasses:

i) Retrovirus-like elements (retrotransposons) which have LTR (long-terminal repeats),
ii) Retroposons which lack LTR; this subclass includes what are called LINEs (long interspersed nuclear elements).

Class II Elements which utilise a DNA to DNA transpositioning mechanism. Here, too, there are two subclasses:

i) Elements with short, inverted terminal repetitions (ITR),
ii) Elements with terminal repetitions of varying length.

There have been many reports of horizontal transfer with the help of eucaryotic transposable elements, especially after the PCR method became universally available. Many offer strong indications of horizontal transfer, but most lack ultimate proof (Kidwell, 1993; Cummings, 1994).

For class I mobile elements a complete picture is obtained of an evolution based on individual modules. Recombinations can concern individual modules or entire elements. The picture also includes DNA fragments that are able to travel from genome to genome across large taxonomic distances, as of course is also clearly documented for retroviruses (Toh *et al*., 1985). The consequence of the module picture will be that phylogenes constructed for different individual genes within a mobile element will be incongruous in relation to one another. This implies recombinations between different species, replacement of an original gene with a homologous foreign gene, or independent gene sorting (McClure, 1991).

It thus seems that horizontal shuffling of genes and portions of genes has occurred between both closely and distantly related retro-elements (Kidwell, 1993). A good example is certain LINE-like elements which only show a sequence difference of approximately 40% between such different species as lily, maize, man, mouse, silkworm and *Drosophila* (Leeton & Smyth, 1993). Another example is Tyl-copia retroposons which are isolated from fungi, insects, vertebrates and protist species, and is also widespread among plants, often in high copy figures (Kidwell, 1993).

For class II mobile elements with short inverted terminal repetitions there are a number of examples of probable lateral gene transfer (review in Kidwell, 1993), Among these are the almost identical P elements in *Drosophila melanogaster* and *D. willistoni*, two species which diverged more than 50 million years ago. The phenomenon is impossible to explain in any other way than through lateral transfer of genes (Daniels *et al*., 1990).

That *mobile introns* existed was implied by the discovery of high sequence homology in introns located in different genes in various organisms. Group I and group II introns are remarkable because they catalyse their own splicing and consequently function as ribozymes, as well as mobile genetic

elements. These "infectious" introns, as they are called, are phylogenetically widely dispersed, and their dispersal reflects the development of varied and efficient mechanisms. There is no longer any doubt that lateral genetic transfer based on mobile introns has been widespread and still takes place frequently in both plants, insects and mammals, and that it may be of great importance in an evolutionary context (Lambowitz, 1989; Kidwell, 1993; Lambowitz & Belfort, 1993).

"Homing" of group I introns, i.e. transfer from an intron-bearing to an intron-lacking allele of the same gene, is well characterised in both eucaryotes and bacteriophagic systems (Lambowitz & Belfort, 1993). The process takes place with the help of intron-coded, sequence-specific endonucleases. "Homing" of group II introns is somewhat more poorly characterised, but occurs at both the DNA and RNA levels, and is dependent upon reverse transcriptase (Lazowska *et al*., 1994).

Both group I and group II introns are capable of transpositioning to other locations, even across phylogenetic boundaries. The molecular mechanisms for transpositioning and self-splicing are well described in recent review articles (Lambowitz & Belfort, 1993; Saldanha *et al*., 1993). Intron transpositioning utilises a strictly RNA-based mechanism, which thus clearly distinguishes it from the "homing" of both group I and group II introns (Grivell, 1994). Transpositioning is assumed to be the most important cause of horizontal dispersal of both group I and group II introns between distantly related organisms. The fact that group I introns are related in structure and function to viroids, i.e. infectious naked RNA, makes an RNA-based transfer mechanism probable (Johansen & Vogt, 1994).

8.5.2.1 Occurrence of mobile introns

Group I introns have the widest distribution and are found in both procaryotes and eucaryotes (Lambowitz & Belfort, 1993). Most of them have been found in genomes of lower eucaryotes such as fungi, algae, ciliates, amoeba, etc. There they are found in nuclear genomes as well as in mitochondrial (mtDNA) and chloroplastic (ctDNA) genomes (Michel & Westhof, 1990; Vader *et al*., 1994). Group I introns are also found in mtDNA and ctDNA from higher plants, in mtDNA in some multicellular animals, in a few eucaryotic viruses and also in the genomes of bacteria and bacteriophages. Nonetheless, there is a strikingly *sporadic* occurrence in the sense that one taxonomic strain or species may have definite group I introns, while these are absent from other strains within the same species, or from other species within the same genus (Belfort, 1990).

Group II introns comprise the majority of introns in ctDNA and mtDNA in higher plants, and a lesser proportion in mtDNA in fungi. The distribution, presence and absence in strains within the same species or between related organisms, and the presence at different sites in the genome of one species, etc. unambiguously demonstrate the mobility of the elements (Lambowitz & Belfort, 1993). Group II introns have recently been proved to occur in bacteria genomes (Ferat & Michel, 1993). The fact that group II introns and spliceosomal mRNA introns have identical splice chemistry and mechanisms, a convincing structural similarity, and that group II introns are present in bacteria genomes which are assumed to be the direct precursors of eucaryotic organelles, has lent support to the hypothesis of *group II introns being the direct precursors of spliceosomal mRNA introns* (Mattick, 1994). *In that case, an invasion of group II introns from procaryotic to eucaryotic genomes, via symbiotic organelles, has led to the most dramatic change known from horizontal gene transfer,*

namely the establishment of mRNA introns in eucaryotes. All eucaryotes, with the exception of amitochondrial protists, contain mRNA introns. Almost all protein-coded genes in vertebrates (ca. 70,000) contain spliceosomal introns and a few genes have more than one hundred of them. Spliceosomal introns play a notably major role in gene control and cell differentiation, particularly in mammals (Mattick, 1994).

There are many potential *transfer routes which may contribute to horizontal transfer of mobile introns or their open reading frames.* They may be transported naked or in packed DNA, with or without simultaneous DNA replication (Lambowitz & Belfort, 1993). Many bacteria are naturally competent for DNA uptake, or can become so under given environmental conditions. They will then, unless enzymatic restriction barriers prevent it, be able to be transformed by DNA from any source whatsoever (Heinemann, 1991). Conjugation between bacteria is not confined to the same species, but can cross phylogenetic boundaries between, for instance, gram-positive and gram-negative species, and still further. Agrobacteria transfer DNA to the cells in the plant host, and efficient conjugation also occurs between *E. coli* and various kinds of yeast (Stachel & Zambryski, 1989).

Eucaryotic cells can take up DNA by a number of mechanisms, including anastomosis, endosymbiosis, phagocytosis, and also endocytosis based on specific receptors for DNA or for macromolecules (most frequently proteins) which are in complex with DNA, and hence take DNA with them into the cell (Loke *et al*., 1989; Yakubov *et al*., 1989; Lambowitz & Belfort, 1993; Whitton, 1994).

Autonomous introns, or intron-like mitochondrial plasmids, which code for reverse trancriptases, may themselves function as infectious elements and vectors for horizontal transfer between organisms (Lambowitz & Belfort, 1993).

In addition to viruses which have eucaryotic hosts, bacteriophages can also function as vectors for horizontal gene transfer. Based on comparison of DNA sequences, bacterio-phage T4 seems to have a common origin with both bacteria and eucaryotes (Bernstein & Bernstein, 1989). It is tempting to speculate on whether bacteriophages with a broad host spectrum may be vectors for shuffling introns between different organisms (Lambowitz & Belfort, 1993).

8.5.3 Biological vectors for horizontal gene transfer

In bacteria, gene transfer regularly takes place across species boundaries, based on transformation, conjugation, transduction and transpositioning (Mazodier & Davies, 1991), and compared with procaryotes it is assumed that stronger barriers to gene transfer exist in eucaryotes (Kidwell, 1993). Nevertheless, there are strong indications that horizontal gene transfer takes place between eucaryotes across species, family and kingdom boundaries (Stachel & Zambryski, 1989).

It appears to be extremely important to have this relationship investigated because even exceptionally rare cases may have far-reaching, unpredictable and serious ecological consequences (Syvänen 1984, 1985, 1987, 1994).

In a few situations, such as in a close symbiotic relationship, physical proximity between donor and recipient may permit horizontal transfer (Zambryski *et al*., 1989). A striking example here is the

distribution of related nuclear group I introns among lower eucaryotic organisms with a pronounced symbiotic and phagocytic way of life (Vader *et al*., 1994; Brul & Stumm, 1994). However, in most situations a *genetic vector* has to be envisaged in order to achieve a horizontal transfer.

This is, of course, what gene technology is very largely concerned with and dependent upon - genetic shuttles (vectors) to transport DNA sequences between species which, in terms of reproduction, are completely isolated from one another (Kidwell, 1993). It surely should not be too surprising if corresponding mechanisms were active under natural conditions.

A list of the possible, *naturally occurring vectors* would have to include:

- *Transposable elements (TE)*
- *Plasmids*
- *Extrachromosomal nucleated ribosomal DNA*
- *Viruses*
- *Chlamydias*
- *Microplasmas*
- *Spiroplasm*
- *Rickettsias*
- *Bacteria*
- *Protozoans*
- *Nematodes*
- *Fungi*
- *Parasitic arthropods*

It is easy to imagine a genetic element that passes like a relay baton from one type of vector to another while in the course of the race being caught by and established in the host organisms of the vectors. Such 'batons' are particularly obvious in connection with *transposable elements (TE)*.

An example of a recent TE invasion exists in connection with the P element of *D. melanogaster*. This invasion began in laboratory strains in the USA during the 1950's and has since spread rapidly to banana fly populations throughout the world (Capy *et al*., 1994). Rickettsia-like organisms are assumed to be involved as genetic vectors in this horizontal gene transfer of TE between different *Drosophila* species. Horizontal spreading of transposable elements between such distantly related organisms as banana flies, nematodes and fungi has been proved, but the underlying mechanisms are not known (Capy *et al*., 1994).

Transposable elements of the Tc1/mariner family are found in many species of the animal kingdom, including the human. The widespread distribution of this transposon family seems to be the result of horizontal transfer between a large number of species. Mariner elements have, among elsewhere, been found in plants (Jarvik & Lark, 1998), fish (Ivics *et al*., 1998), mosquitoes (Leung & Romans, 1998), lepidoptera and their baculoviruses (Jehle *et al*., 1998), nematodes (Schouten *et al*., 1998), as well as humans and other mammals (McNaughton *et al*., 1997). The mariner transposons easily cross species and kingdom boundaries, for instance from fish to mouse and human cells (Ivics *et al*., 1997; Luo *et al*., 1998) and from a nematode into human cells (Schouten *et al*., 1998). In fact,

natural and reconstructed Tc1/mariner transposons are now being engineered for jumping around between different species. This certainly creates a new ecological problem.

To be able to understand the development and dispersal of TEs, it is necessary to have more information about the elements, the biology and ecology of the host species, and the vectors which may transfer TEs (and other DNA) from one species to another (Capy *et al*., 1994). As vectors, in addition to parasites, it is necessary to think about those that are themselves nucleic acids, i.e. plasmids, viruses with double-stranded DNA or RNA, and single-stranded RNA genomes. Some forms of TE, such as so-called mobile, 'autonomous' introns or intron-like mitochrondrial plasmids, may themselves function as vectors for horizontal gene transfer, particularly because some of them have open reading frames which code for reverse transcriptases (Lambowitz & Belfort, 1993).

Some *viruses* have a limited host spectrum. There will then be little likelihood that *infection* will take foreign DNA picked up by the virus across species boundaries, whereas such transfer may nevertheless take place by natural *transfection* of naked viral nucleic acids. Other viruses, for instance within the ecological grouping, arboviruses, easily cross species boundaries. Some arboviruses may even alternate between vertebrates and blood-sucking arthropods as an essential part of their life cycle (Anderson, 1970; Traavik, 1979; Syvänen, 1987).

Proof exists that *retroviruses* are able to function as transfer vectors between different mammals (Duesberg, 1983) and that host-cell mRNA can be carried from one vector to another (Ikawa *et al*., 1974). Retroviruses may contribute significantly to transduction of genetic material between mammalian cells and individuals (Coffin, 1990). This possibility is made more likely by the use of retrovirus vectors in gene therapy and to establish transgenic animals. Insertions from such vectors into the DNA virus which infect the same individuals or organs may result in recombinant virus being released from the individual that is treated, taking both the retrovirus and the target gene sequences to new individuals. There are many examples that retrovirus DNA really can be integrated into the genome of DNA viruses when a co-infection of a common host cell takes place. The phenomenon has been observed in experimental model trials, but also in natural isolates of viruses within the *Herpes* and *Papova viridae* families (review in Isfort *et al*., 1994). The recombination process is usually steered by the retroviral integrase, but host cell recombinases may also be involved (Sun *et al*., 1993).

There are good indications that *baculoviruses*, which are now used in recombinant versions as laboratory tools and for combating insect pests, have spread mobile elements between various insect host species (Miller & Miller, 1982; Friesen & Nissen, 1990; Jehle *et al*., 1995). Some baculovirus species have proved to contain transposable elements from their host organisms. Since the same virus species may have several host species, this implies the possibility for spreading recombinant DNA between the host species, too. The transposable elements can, of course, have picked up foreign genes from, for example, a GMO or a plasmid which was located in a particular host species (Capy *et al*., 1994). In this connection, it is also worth noting that baculoviruses, originally claimed to be strictly insect-related, have now been proven to infect human cells. In fact, these viruses are now considered highly efficient vectors for gene therapy applications (Boyce & Bucher, 1996; Hofmann & Strauss, 1998).

Very recently, total sequencing of the *Chlamydia trachomatis* genome has brought strong indications that this intracellular parasite has picked up a large number of genetic elements from eucaryotic host organisms (Stephens *et al*., 1998).

Arthropods, particularly ectoparasites (mites, parasitic wasps, biting midges, etc.), may function as living injection needles and transfer DNA (plasmids, TEs, other types of naked nucleic acids) between their host species. They can obviously also themselves receive foreign sequences in that manner (Capy *et al*., 1994). Recently, a very interesting potential arthropod vector for lateral gene transfer has been identified, *Proctolaelaps regalis*, a small mite which parasites *Drosophila* species (Houck *et al*., 1991). There seems to be convincing evidence that this mite functions as a mechanical vector for *Drosophila* DNA. Whether this is a unique case of an arthropod vector, or is only the first one to be discovered, is still a completely open question.

9. Do pollution and other environmental changes affect the horizontal transfer or negative impacts of naked DNA?

Very little work seems to have been done with regard to how xenobiotics may interfer with horizontal gene transfer under natural conditions or in microcosm and other types of controlled experiments. However, a vast literature concerning other effects of environmental pollutants indicates that such effects may exist (e.g. Darbree, 1998; Ferguson, 1998; Zacharewski, 1998; Smital & Kurelec, 1998; Wirgin & Waldman, 1998; Steinmetz *et al*., 1998; Tyler *et al*., 1998; Williams *et al*., 1998).

Xenobiotics are, literally, compounds that are alien in the biosphere. Nevertheless, with such a narrow definition, metals, some pesticides and many organic chemicals would not be considered xenobiotics because they are also found naturally in ecosystems. The definition does not take human activity into account, which may increase the concentration of natural compounds to levels which give damaging effects. The essential element phosphorous is a good example. It is not usually a xenobiotic compound, but in large amounts we know it can create major environmental problems. Consequently, the following definition is used: *xenobiotics are compounds which people release into Nature in concentrations that create undesirable impacts.*

Different xenobiotics have properties and biological activities that enable us to envisage *at least two different sets of possible impacts* on the fate of naked DNA in an ecosystem.

i) Some xenobiotics can act as mutagenes (this applies to both radioactive substances, polluting industrial chemicals and plant protectants). Mutagenes can result in naked DNA that escapes or is released having its sequence or structure changed. This, in turn, can affect the possibilities for DNA uptake in cells and organisms, horizontal transfer and long-term establishment in the ecosystems in ways which we are totally incapable of predicting. Kipling & Kearsey (1990) have reported examples of minor changes in a DNA sequence altering the host spectrum of a transferable genetic element.

ii) Some xenobiotics can affect cell membrane and/or intracellular functions in ways which can very well be thought to influence the ability of cells to take up and horizontally transfer naked DNA. This concerns the structure of cell membranes and the content of both surface receptors and transport canals, and also for intracellular signal conversion and gene expression. For instance, xenobiotics which mimic hormones or affect the local conditions in the organ systems of mammals (e.g. respiratory passages) may change the possibilities for both uptake and establishment of foreign nucleic acids in animals and people.

Some xenobiotics will be found in both categories, and we do not know how the sum of the impacts of such substances will turn out. Likewise, up to several individual compounds from each category will often pollute the same environment. We have no knowledge of how such situations affect DNA uptake and dispersal in the ecosystems.

Many of the xenobiotics with which man has polluted his environment during the past decades (e.g. herbicides, pesticides, heavy metals, emissions from industry and the burning of fossil hydrocarbons, etc.) have in common that they are chemically inert and hydro-phobic. *Hydrophobism* means that they easily enter organisms by diffusing through biological membranes, are difficult to separate in urine and gall, and accumulate in certain areas of the cell, including the phosphorous-lipid double layer in the membranes where they are able to disturb normal cellular functions (Lundgren & DePierre, 1990).

Most organisms have natural *inactivation mechanisms for xenobiotics*, but the efficiency in mammals may, on a genetic basis, vary several hundred times between separate individuals of the same species (Lundgren & DePierre, 1990). The biochemical processes between closely related species of fish may also vary so much that one species develops liver cancer through a concentration of polycyclic aromatic hydrocarbons which does not affect the other species (Stein *et al*., 1990). A number of intermediaries from the breakdown of xenobiotics which are not inactivated sufficiently quickly may attach covalently to both RNA, DNA and proteins, and such attachment may lead to both toxic and teratogenic effects, carcinogenesis and mutagenesis (Lundgren & DePierre, 1990; el-Bayoumy *et al*., 1994).

Xenobiotics are obviously, in practice, never found one at a time in the environment. Interactions between several xenobiotics will probably affect organisms and cells differently from a single substance alone (Hicks *et al*., 1990). It is therefore surprising that relatively few studies are found which take this into account. Whereas antagonistic effects between different contaminating chemicals have been investigated for many years, it is first quite recently that *synergistic* effects have been studied. However, a number of articles in the last few years have demonstrated that the synergistic effects are unpredictable. For instance, severe *mutagenic synergism* has been proved between different kinds of pesticides, between pesticides and X-rays, between heavy metals and radioactivity, etc. (Shima & Ichikawa, 1994; Lee *et al*., 1994; Newman, 1995).

Synergism may mean that far lower concentrations of the individual xenobiotics have biological activity and the absolute minimum values vary according to which other forms of contamination are found. It may also mean that even the phenotypical evidence or symptoms at low concentrations of individual xenobiotics are changed. This is an enormous field of research which, for the moment, is almost untouched in any context, and not least as regards the ecological risks of the release of recombinant DNA.

Data bases contain good review articles about the ability of micro-organisms to break down xenobiotics, and how the life processes of micro-organisms are affected by some xenobiotics (see, for example, Ghiorse & Wilson, 1988; Cork & Krueger, 1991; Stotzky *et al*., 1993). However, it is difficult to find *references concerning how xenobiotics affect the competence of organisms for uptake of naked DNA, permissiveness for viruses, ability for conjugation, etc.)*. This is curious, because the types of cellular functions that are usually affected by xenobiotics, such as the composition and permeability of cell membranes, the synthesis of nucleic acids and proteins, etc., can very well be envisaged to have an impact on the possibility for horizontal transfer of genes. The extent to which xenobiotics affect living cells and organisms depends upon the specific physico-chemical conditions, such as the type of soil, the temperature, the water content and the pH, factors which, in turn, may be affected by other types of contamination, local emissions, etc. (Hicks *et al*., 1990).

The various methods employed to make bacteria competent for genetic transformation under laboratory conditions can be divided into the following main groups (Mercenier & Chassy, 1988): i) treatment with solutions of calcium chloride or chlorides of other elements, including magnesium, barium, rubidium, strontium and mixtures of such heavy metals; ii) treatment with EDTA or other chelating aids; iii) treatment with enzymes (e.g. muraminidases or proteases); iv) fusion of cells with DNA, with other cells, or with DNA packed in liposomes; v) freezing and thawing of cells; vi) exposure of cells to electric fields; vii) bombardment of cells with small particles which transport DNA into cytoplasm (biolistic transformation).

It is not difficult to imagine that bacteria in ecosystems can be exposed to conditions that coincide with the laboratory conditions listed above, and even to several at a time. The concentrations of heavy metals may vary within quite wide extremes over time and from place to place. The same applies to phosphate emissions, and it is known from laboratory experiments that enhanced phosphate concentration may reduce the thickness of the capsules of some bacteria, thereby increasing their competence for DNA uptake (Page, 1985). *How variations in pH, for instance in the form of acid precipitation, will affect the uptake of naked DNA in micro-organisms, plants and animals is in reality completely unknown* (see also section 8.4).

A large number, and large amounts, of chemicals which *mimic or interrupt the mechanisms of hormones* have been released into the environment since the Second World War. If vertebrates are exposed in the foetal state or after birth, many of these chemicals can disturb the development of the important glandular systems (endocrinal organs) of the body, and thereby of the other organs that are dependent upon correctly tuned hormone signals for normal development and function. Such effects on single individuals are permanent and irreversible. Effects which span over generations can arise as a consequence of the exposure of a female to chemicals at any moment in her life before she produces offspring. This is due to the storage of the hormone-imitating chemicals in her body fat. These are mobilised during egg laying or pregnancy. More than 50 chemicals that are widely dispersed over the entire globe, herbicides, fungicides, insecticides, nematocides, industrial chemicals such as dioxin, PCB and phenols, as well as metals such as cadmium, lead and mercury, can act as hormone imitators (Colborn *et al*., 1994).

On the cellular level, hormone-imitating xenobiotics will be capable of disturbing the transfer of signals from the cell surface to the nucleus, and hence the genetic expression of the cell. We know nothing about how this affects the opportunities for foreign DNA to be taken up and few of the biological consequences in eucaryotic cells and organisms.

One of the most cunning threats to which the environment and public health are now exposed is the group of chlorinated hydrocarbons which goes under the collective name of ***dioxins***. This group includes dioxin proper, TCDD (2,3,7,8 tetrachlorodibenzo-*p*-dioxin, and the closely related compounds CDD (chlorinated dibenzodioxins), CDF (chlorinated dibenzofurans) and PCB (polychlorinated biphenyls).

The great biological potential and the fundamental level in living cells which the dioxins act upon are analogous to many well-studied steroid hormones. However, *the individual dioxins can either enhance the effect of naturally occurring hormones or counteract them*. The dioxins have the ability

of changing the growth pattern and differentiation programmes of a large number of target cells by initiating biochemical and biological processes which may give a whole range of responses in animals and humans (U.S. Environmental Protection Agency, 1994a).

The presence of the dioxins in the environment is quite obviously, and without doubt, due to man-made pollution. Incinerating plants and industrial emissions are the greatest sources. The primary mechanism for the entry of the dioxins into food chains is precipitation from the atmosphere onto plants and the soil. Humans are exposed through intake of food containing small amounts of dioxins. The most important sources are fatty dairy products, fish and meat (U.S. Environmental Protection Agency, 1994b, 1994c).

A series of common biological steps have been identified and described that are preconditions for most, perhaps all, of the effects of dioxins observed on vertebrates, including man. The first step is the binding of dioxin to the intracellular protein called the Ah receptor. This receptor is a gene-regulating protein which displays many similarities common with steroid hormone receptors. Activation of the receptor is a two-stage process that comprises the binding of dioxin and dissociating of hsp90 from the receptor protein. When the receptor is activated in this manner it can attach itself to XRE (xenobiotic response element) sequences in cellular promotors and enhancers, and alter the genetic expression of cellular genes (Wilhelmson *et al*., 1990).

When one is working with a receptor model, where xenobiotics imitate or interfere with natural compounds in living organisms, the effect can give several endpoints. Bimodal responses can be observed, depending upon the length of exposure, age and sex. In the earliest DDT trials, high doses were used and, for example, thinner egg shells were not found in bird species whereas the natural effects on them later proved to be catastrophic. Important impacts can thus be overlooked when high-dosage tests are used. In the short term, the effects concerned may be subtle and occur in the form of altered or reduced functions, whereas dramatic changes will not be seen which increase mortality or serious malformations. Dioxin-mediated changes of genetic expression may have several other consequences that have been recognised in animals for many years, and the available literature strongly indicates that people respond in the same way. A large number of foetus-developing programmes can be disturbed. This has been proved for several species within three classes of vertebrates. Negative impact on the reproductive ability of both masculine and feminine individuals is well documented, and there is a clear connection between exposure to dioxins and increased cancer mortality. Many of the functional disturbances, especially with regard to impact on foetuses, occur at nanogram and picogram concentrations, just as in the case of natural hormones (Colborn, 1994; Environmental Impact Assessment Review Team, 1994). The same is the case for effects which influence sexual traits and fertility. The impact of such properties may already be in process of attaining epidemic proportions in animals and people without us having so far acknowledged it (Colborn, 1994). There is a continuum of responses on the exposure of organisms to dioxin-like chemicals. As the total load increases, the probability for individual impacts and the degree of collective effects increase. On the basis of such a continuum, we have opportunities for acknowledging the link between early effects which are not necessarily realised as damaging, and late effects which definitely are damaging (U.S. Environmental Protection Agency, 1994a, b, c).

10. Conclusion and recommendations

"The problem is that we've always worked from the wrong end. We've always looked for very gross and obvious endpoints, such as cancer and mortality".

Theo Colborn

Too early may be too late simply because we know too little. It is quite obvious that the horizontal transfer of genes is a natural process with positive effects for evolution, and there are strong selection pressures and functional barriers that decide which kinds of nucleic acids are to be transferred and which must absolutely not be transferred. However, we know too little about what characterises nucleic acids that *are* horizontally transferred, established and spread across species boundaries, ecosystem boundaries and great geographical distances, as well as about the mechanisms that convey them. Likewise, we know too little about the mechanisms that create barriers to transfer. Little is being done to investigate these mechanisms. On the other hand, a great deal of research effort is being directed towards making recombinant DNA, anti-sense oligonucleotides and ribozymes more resistant to biological breakdown. This is very important research because if such constructs continue to function for longer periods they can provide a basis for saving many human lives through enhanced opportunities for gene therapy and vaccination. However, too little attention is being paid to the fact that these same constructs may have had their potential for negative ecological impacts strengthened. The more efficient they are for their intended use, the more dangerous they may be when they are released. This exemplifies the cross-pressure situations that arise in the use of recombinant DNA, just as with all other technology. The positive effects are obvious, and available here and now. The pressure to rapidly make use of them will be great. Negative consequences are theoretical and often long term. Hence, relatively little emphasis is put on them and it is, moreover, believed that technological solutions will be found for these problems, too.

The useful genetic engineering products and processes that are continually being developed are achieved by the effort of many research groups in universities and the business sector throughout the world. Huge investments of talent, enthusiasm, work effort, economic resources and, in many cases, idealism are required.

It is *not* desirable that such groups should split up their creativity and resources to investigate negative ecological impacts. It is simply not possible for the same group to work hard and enthusiastically to achieve more stable DNA constructs, better and more long-term genetic expression, etc., and at the same time worry about, and show an equally great ability for innovation in the investigation of potentially damaging impacts. These groups must be allowed to continue with what they have already proved themselves capable of.

Research directed at understanding general mechanisms for horizontal transfer of genes, which aim to strengthen the barriers to the transfer of recombinant DNA, or which can give leads for preassessing risks, must be carried out by independent research groups which have this important new

field as their principal pursuit. At the moment, there is scarcely a single such research group in the world. Such groups must now be built up from the bottom, both nationally and internationally. National groups must exist because local and regional conditions greatly influence the fate of the nucleic acids in the ecosystems.

This is a field where Norway is well qualified to become a pioneer nation if it acts in time. We have the necessary scientific and technological capabilities, and we have independent base institutions and scientists to form the foundation for a strong, national research centre. The establishment of such a research centre must be a public responsibility, but it must be a declared aim that the centre must rapidly achieve results and a reputation that permits it to compete advantageously for research funds both nationally and internationally.

This proposal certainly does not have its background in any kind of lack of confidence in existing commercial and academic research groups. The key words must be functional division of labour and confidential co-operation. If *this* is translated into practice, the academic and industrial gene technology milieus and the new molecular ecology milieus will be able to mutually fortify one another both intellectually and methodologically. The overriding objective is, of course, the same: to utilise technology to the advantage of mankind without compromising the spaceship Tellus.

References

Acsadi, G. *et al*. 1991. Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs. Nature 352: 815-818.

Adamo, J.A. & Gealt, M.A. 1996. A demonstration of bacterial conjugation within the alimentary canal of *Rhabditis* nematodes. FEMS Microbiol. Ecol. 20: 15-22.

Ahrenholz, I. *et al*. 1994. The extracellular nuclease of *Serratia marcescens*: studies on the activity *in vitro* and effect on transforming DNA in a groundwater aquifer microcosm. Arch. Microbiol. 161: 176-183.

Akhtar, S. 1998. Antisense technology: selection and delivery of optimally acting antisense oligonucleotides. J. Drug Target 5: 225-234.

Albritton, W.L. *et al.* 1984. Heterospecific transformation in the genus *Haemophilus*. Mol. Gen. Genet. 193: 358-363.

Andersen, S.R. & Sandaa, R.A. 1994. Distribution of tetracycline resistance determin-ants among gram-negative bacteria isolated from polluted and unpolluted marine sedi-ments. Appl. Environ. Microbiol. 60: 908-912.

Anderson, J.R. & Roth, J.R. **1981.** Spontaneous tandem genetic duplications in *Salmonella typhimurium* arise by unequal recombination between rRNA (rrn) cictrons. Proc. Natl. Acad. Sci. USA 78: 3113-3117.

Anderson, J.R. 1987. Twenty years of illegitimate recombination. Genetics 115: 581-584.

Anderson, N.G. 1970. Evolutionary significance of virus infection. Nature 227: 1346-1347.

Anderson, R.P. & Roth, J.R. 1977. Tandem genetic duplication in phage and bacteria. Annu. Rev. Microbiol. 31: 473-505.

Antebi, E. & Fishlock, D. 1986. *"Biotechnology: strategies for life."* MIT Press, Cambridge, Massachusetts.

Arber, W. 1984. Natural mechanisms of microbiol evolution. Pp. 1-14 in: Arber, W. *et al*. (eds.). *Genetic manipulation: Impact on Man and Society*. 3rd Cogene Symposium. Cambridge University, Cambridge.

Australian Gen-Ethics Network 1994. *The troubled helix. Vol. 3* (ISBN 0-85802-115-3 08502-095-5).

Bakker, E. *et al*. 1987. Germline mosaicism and Duchenne muscular dystrophy mutations. Nature 329: 554-556.

Barlow, N.D. 1994. Predicting the effect of a novel vertebrate biocontrol agent: a model for viral-vectored immunocontraception of New Zealand possums. J. Appl. Ecol. 31: 454-462.

Barry, M.A. *et al*. 1994. Production of monoclonal antibodies by genetic immunization. BioTechniques 16: 616-620.

Basse, G. *et al*. 1995. A biological assay for the sensitive and quatifiable detection of extracellular microbial DNases. J. Microbiol. Methods.

Belfort, M. 1990. Phage T4 introns: selfsplicing and mobility. Annu. Rev. Gen. 24: 363-385.

Berg, D.E. & Howe, M.M. (eds.) 1989. Mobile DNA. Am. Soc. for Microbiol., Washington D.C.

Bergan, T. & Vaksvik, A.K. 1983. Taxonomic implications of quantitative transforma-tion in *Acinetobacter calcoaceticus*. Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. A. 254: 214-228.

Bergelson, J. *et al*. 1998. Promiscuity in transgenic plants. Nature 395: 25.

Bernstein, H. & Bernstein, C. 1989. Bacteriophage T4 genetic homologies with bacteria and eucaryotes. J. Bacteriol. 171: 2265-2270.

Bird, A.P. 1995. Gene number, noise reduction and biological complexity. Trends Genet. 11: 94-100.

Bogosian, G. & Kane, J.F. 1991. Fate of recombinant *Escherichia coli* K-12 strains in the environment. Adv. Appl. Microbiol. 36: 87-131.

Borie, D.C. *et al*. 1998. Microbiological hazards related to xenotransplantation of porcine organs into man. Infect. Hosp. Epidemiol. 19: 355-365.

Bork, P. & Doolittle, R.F. 1992. Proposed acquisition of an animal protein domain by bacteria. Proc. Natl. Acad. Sci. USA. 89: 8990-8994.

Boxhorn, H.K. & Eck, S.L. 1998. Gene therapy for breast cancer. Hemat. Oncol. Clin. North Am. 12: 665-675.

Boyce, F.M. & Bucher, N.L. 1996. Baculovirus-mediated gene transfer into mammalian cells. Proc. Natl. Acad. Sci. USA 93: 2348-2352.

Branch, A.D. 1998. A good antisense molecule is hard to find. Trends Biochem. Sci. 23: 45-50.

Brown, L.R. (ed.) 1997. *State of the world*. Worldwatch Institute, Washington, Norsk utgave: *Jordens tilstand*. H. Aschehoug & Co (W. Nygaard), Oslo. (ISBN 83-03-22190-4).

Brul, S. & Stumm, C.K. 1994. Symbionts and organelles in anaerobic protozoa and fungi. Trends Ecol. Evol. 9: 319-323.

Bruns, S. *et al*. 1992. Characterization of natural transformation of the soil bacteria *Pseudomonas stutzeri* and *Acinetobacter calcoaceticus* by chromosomal and plasmid DNA. Pp. 115-126 in: Gauthier, M.J. (ed.) *Gene transfers and environment*. Springer Verlag, Berlin.

Byrd, J.J. *et al*. 1992. Determination of plasmid DNA concentration maintained by nonculturable *Escherichia coli* in marine microcosms. Appl. Environ. Microbiol. 58: 2266-2270.

Børsheim, K.Y. 1993. Native marine bacteriophages. FEMS Microbiol. Ecol. 102: 141-159.

Caddle, M.S. & Calos, M.P. 1992. Analysis of the autonomous replication behavior in human cells of the dihydrofolate reductase putative chromosomal origin of replication. Nucl. Acids Res. 20: 5971-5978.

Cairns, J. jr. & Orvos, D.R. 1992. Established environmental hazards of genetically engineered microorganisms. Rev. Environ. Contam. Toxicol. 124: 19-39.

Cameron, J. & Abouchar, J. 1991. *The precautionary principle: A fundamental*

Canonico, A.E. *et al*. 1994. Aerosol and intravenous transfection of human alpha 1-antitrypsin gene to lungs of rabbits. Am. J. Respir. Cell. Mol. Biol. 10: 24-29.

Capy, P. *et al*. 1994. The strange phylogenies of transposable elements: are horizontal transfers the only explanation? TIG 10: 7-12.

Carlson, C.A. *et al*. 1983. *Pseudomonas stutzeri* and related species undergo natural transformation. J. Bacteriol. 153: 93-99.

Carson, S. & Wiles, M.V. 1993. Far upstream regions of class II MHC Ea are necessary for position-independent, copy-dependent expression of Ea transgene. Nucl. Acids Res. 21: 2065-2072.

Caselmann, W.H. *et al*. 1997. Synthetic antisense oligonucleotides as potential drugs against hepatitis C. Intervirology 40: 394-399,

Chamier, B. *et al*. 1993. Natural transformation of *Acinetobacter calcoaceticus* by plasmid DNA adsorbed on sand and groundwater aquifer material. Appl. Environ. Microbiol. 59: 1662-1667.

Chatterjee, S. *et al*. 1992. Dual target inhibition of HIV-1 *in vitro* by means of an adeno-associated virus antisense vector. Science 258: 1485-1488.

Chen, J. *et al*. 1994. A novel gene delivery system using EGF receptor-mediated endocytosis. FEBS Letters 338: 167-169.

Cheng, L. *et al.* 1993. *In vivo* activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. Proc. Natl. Acad. Sci. USA 90: 4455-4459.

Chévre, A.M. *et al.* 1997. Gene flow from transgenic crops. Nature 389: 924.

Chivian, E. *et al.* (eds.) 1993. *Critical condition: human health and the environment*. P. 2. MIT Press, Cambridge, Massachusetts.

Clegg, M.T. *et al.* 1997. The evolution of plant nuclear genes. Proc. Natl. Acad. Sci. USA 94: 7791-7798.

Clerc, S. & Simonet, P. 1998. A review of available systems to investigate transfer of DNA to indigenous soil bacteria. Antonie van Leeuwenhoek 73: 15-23.

Coffin, J. 1990. Pp. 1437-1527 in: Fields, B. *et al*. (eds.) *Virology*. Raven Press.

Cohan, F.M. *et al.* 1991. The potential for genetic exchange by transformation within a natural population of *Bacillus subtilis*. Evolution 45: 1383-1421.

Cohen, J. 1993. Naked DNA points way to vaccines. Science 259: 1691-1692.

Cohen, S.N. *et al.* 1973. Construction of biologically functional plasmids *in vitro*. Proc. Natl. Acad. Sci. USA. 70: 3240-3244.

Colborn, T. *et al.* 1994. Developmental effects of endocrine-disrupting chemicals in wild-life and humans. Environ. Impact Assess. Rev. 14: 469-489.

Coleman, J. *et al.* 1984. The use of RNAs complementary to specific mRNAs to regulate the expression of individual bacterial genes. Cell 37: 429-436.

Cork, D.J. & Krueger, J.P. 1991. Microbial transformations of herbicides and pesticides. Adv. Appl. Microbiol. 36: 1-66.

Cortese, A.D. 1993. Human health, risk and the environment. P. 2 in: Chivian, E. *et al.* (eds.) *Critical condition: human health and the environment*. MIT Press, Cambridge, Massachusetts.

Crabb, W.D. *et al.* 1977. Selective enrichment for genetic markers in DNA released by competent cultures of *Bacillus subtilus*. Mol. Gen. Genet. 155: 179-183.

Croce, C.M. 1987. Role of chromosome tranlocations in human neoplasia. Cell 49: 155-156.

Crooke, R.M. *et al.* 1991. *In vitro* toxicology and pharmokinetics of antisense oligo-nucleotides. Anticancer Drug Res. 6: 609-646.

Cummings, M.P. 1994. Transmission patterns of eucaryotic transposable elements: arguments for and against horizontal transfer. Trends Ecol. Evol. 9: 141-145.

Daane, L.L. *et al*. 1997. Influence of earthworm activity on gene transfer from *Pseudo-monas fluorescens* to indigenous soil bacteria. Appl. Environ. Microbiol. 62: 515-521.

Daniels, S.B. *et al*. 1990. Evidence for horizontal transmission of the P transposable element between *Drosophila* species. Genetics 124: 339-355.

Darbree, P.D. 1998. Environmental contaminants in milk: the problem of organochlorine xenobiotics. Biochem. Soc. Trans. 26: 106-112.

Davis, H.L. *et al*. 1991. DNA-based immunization induces continuous secretion of hepatitis B surface antigen and high levels of circulating antibodies. Hum. Mol. Gen. 2: 1847-1851.

DeFlaun, M.F. & Levy, S.B. 1989. Genes and their varied hosts. Pp. 1-32, in: Levy, S.B. & Miller, R.V. (eds.) *Gene transfer in the environment*. McGraw-Hill, New York.

Dellaire, G. & Chartrand, P. 1998. Direct evidence that transgene integration is random in murine cells, implying that naturally occurring double-strand breaks may be distributed similarly within the genome. Radiat. Res. 149: 325-329.

DeLey, J. 1992. The proteobacteria: ribosomal RNA cistron similarities and bacterial taxonomy. Pp. 2111-2140 in: Balows, A. *et al*. (eds.) *The prokaryotes*. Springer Verlag, New York.

Dilber, M.S. 1998. Gene transfer into hematopoietic cells: progress, problems and prospects. Turk. J. Pediatr. 40: 307-336.

Doerfler, W. & Schubbert, R. 1998. Uptake of foreign DNA from the environment: the gastrointestinal tract and the placenta as portals of entry. Wien Klin. Wochenschr. 110: 40-4.

Doerfler, W. *et al*. 1997. Integration of foreign DNA and its consequences in mammalian systems. TIBTECH 1997; 312: 401-6.

Doolittle, R.F. *et al*. 1989. Origins and evolutionary relationships of retroviruses. Q. Rev. Biol. 64: 1-29.

Dorward, D.W. & Garon, C.F. 1990. DNA is packaged within membrane-derived vesicles of gram-negative bt not gram-positive bacteria. Appl. Environ. Microbiol. 56: 1960-1962.

Dreiseikelmann, B. 1994. Translocation of DNA across bacterial membranes. Microbiol. Rev. 58: 293-316.

Dubnau, D. 1991. Genetic competence in *Bacillus subtilus*. Microbiol. Rev. 55: 395-424.

Duesberg, P.H. 1983. Retroviral transforming genes in normal cells. Nature 304: 219-226.

Ehrlich, S.D. 1989. Illegitimate recombination in bacteria. Pp. 799-832 in: Berg, D.E. & Howe, M.M. (eds.) *Mobile DNA*. Am. Soc. Microbiol. Washington D.C.

Ehrlich, S.D. *et al*. 1986. Structural instability of *Bacillus subtilis* plasmids. Pp. 71-83 in: Setlow, J.K. & Hollaender, A. (eds.) *Genetic engineering, Vol. 8.* Plenum Press, New York.

Ehrlich, S.D. *et al*. 1993. Mechanisms of illegitimate recombination. Gene 135: 161-166.

el-Bayoumy, K. *et al*. 1994. Biomonitoring of nitropolynuclear aromatic hydrocarbons via protein and DNA adducts. Health Effects Institute Research Report 64: 1-27.

Environmental Impact Assessment Review Team 1994. An interview with Theo Colborn. Environ. Impact Assess. Rev. 14: 491-497.

Falk, B.W. & Bruening, G. 1994. Will transgenic crops generate new viruses and new diseases? Science 263: 1395-1396.

Farr, C.J. *et al*. 1995. Generation of a human X-derived minichromosome using telomere-associated chromosome fragmentation. EMBO J 14: 5444-5454.

Favrot, M. *et al*. 1998. Cell death and cancer: replacement of apoptotic genes and inactivation of death suppressor genes in therapy. Gene Therapy 5: 728-739.

Felgner, J.H. *et al*. 1994. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J. Biol. Chem. 269: 2550-2561.

Ferat, J.L. & Michel, F. **1993.** Group II self-splicing introns in bacteria. Nature 364: 358-361.

Ferguson, L.R. 1998. Inhibitors of topoisomerase II enzymes: a unique group of environmental mutagens and carcinogens. Mutat. Res. 400: 271-278.

Fox, J.L. 1997. Farmers say Monsanto´s engineered cotton drops bolls. Nature Biotechnol. 15: 1233.

Fredriksen, K. 1993. *Anti-native DNA antibodies induced by BK virus and BK virus native DNA*. Ph.D. thesis, Department of Virology, Institute of Medical Biology, University of Tromsø, Norway (ISBN 82-7589-030-6).

Fredriksen, K. *et al*. 1994. On the biological origin of anti-double stranded (ds) DNA antibodies: systemic lupus erythematosus-related anti-dsDNA antibodies are induced by polyomavirus BK in lupus-prone (NZB x NZW) F1 hybrids, but not in normal mice. Eur. J. Immunol. 24: 66-70.

Friedman, T. & Roblin, R. 1972. Gene therapy for human genetic disease? Science 178: 648-649.

Friesen, P.D. & Nissen, M.S. 1990. Gene organization and transcription of TED, a Lepidoptera retroposon integrated within the baculovirus genome. Mol. Cell Biol. 10: 3067-3077.

Frischer, M.E. *et al.* 1993. Factors effecting competence in a high frequency of trans-formation marine Vibrio. J. Gen. Microbiol. 139: 753-761.

Fung, J. *et al.* 1997. Clinical trials and projected future of liver xenotransplantation. World J. Surg. 21: 956-961.

Fynan, E.F. *et al.* 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. USA 90: 11478-11482.

Gao, L. *et al.* 1993. Direct *in vivo* gene transfer to airway epithelium employing adenovirus-polylysine-DNA complexes. Hum. Gene Therapy 4: 17-24.

Garcia, E. *et al.* 1978. Early stages in *Bacillus subtilis* transformation: association between homologous DNA and surface structures. J. Bacteriol. 135: 731-740.

Gebauer, G. *et al.* 1998. mRNA expression of components of the insulin-like growth factor system in breast cancer cell lines, tissues, and metastatic breast cancer cells. Anti-cancer Res. 18:2A 1191-5.

Ghiorse, W.H. & Wilson, J.T. 1988. Microbial ecology of the terrestrial subsurface. Adv. Appl. Microbiol. 33: 107-172.

Gilboa, E. & Smith, C. 1994. Gene therapy for infectious diseases: the AIDS model. TIG 10: 139-144.

Goodgal, S.H. 1982. DNA uptake in *Haemophilus* transformation. Annu. Rev. Genet. 16: 169-192.

Gorbunova, V. & Levy, A.A. 1997. Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions. Nucleic Acid Res. 25: 4650-4657.

Gorman, T. 1994. "Killer bee" swarm crosses into state. Los Angeles Times, November 1, 1994.

Graham, J.B. & Istock, C.A. 1978. Genetic exchange in *Bacillus subtilis* in soil. Mol. Gen. Genet. 166: 287-290.

Graham, J.B. & Istock, C.A. 1979. Gene exchange and natural selection cause *Bacillus subtilis* to evolve in soil culture. Science 204: 637-639.

Graham, J.B. & Istock, C.A. 1981. Parasexuality and microevolution in experimental populations of *Bacillus subtilis*. Evolution 35: 954-963.

Greaves, M.P. & Wilson, M.J. 1970. The degradation of nucleic acids and montmorillonite-nucleic acid complexes by soil microorganisms. Soil Biol. Biochem. 1: 317-323.

Greene, A.E. & Allison, R.F. 1994. Viruses and transgenic crops. Science 263: 1423-1424.

Greene, A.E. & Allison, R.F. 1996. Deletions in the 3′ untranslated region of cowpea chlorotic mottle virus transgene reduce recovery of recombinant viruses in transgenic plants. Virology 225: 231-4.

Griffith, F. 1928. The significance of pneumococcal types. J. Hyg. 27: 113-159.

Grivell, L.A. 1994. Invasive introns. Curr. Biol. 4: 161-164.

Grosvold, F. *et al.* 1987. Position-independent, high-level expression of the human b-globin gene in transgenic mice. Cell 51: 975-985.

Haas, R. *et al.* 1993. Aflagellated mutants of *Heliobacter pylori* generated by genetic transformation of a naturally competent strain using transposon shuttle mutagenesis. Mol. Microbiol. 8: 753-760.

Hamlin, J.L. *et al.* 1992. Mammalian origins of replication. BioEssays 14: 651-659.

Han, L. *et al.* 1991. Inhibition of Moloney murine leukemia virus-induced leukemia in transgenic mice expressing antisense RNA complementary to the retroviral packaging sequences. Proc. Natl. Acad. Sci. USA 88: 4313-4317.

Hansen, E. *et al.* 1991. Strong expression of foreign genes following direct injection into fish muscle. FEBS Lett. 290: 73-76.

Hanvey, J.C. *et al.* 1992. Antisense and antigen properties of peptide nucleic acids. Science 258: 1481-1485.

Harding, K. 1996. The potential for horizontal gene transfer within the environment. Agro Food Ind. Hi-Tech. 7: 31-35.

Harford, N. & Mergeay, M. 1973. Interspecific transformation of rifampicin resistance in the genus *Bacillus*. Mol. Gen. Genet. 120: 151-155.

Hawkinson, S.E. *et al.* 1998. Circulating concentrations of insulin-like growth factor 1 and risk of breast cancer. Lancet 351: 1393-6.

Heinemann, J.A. 1991. Genetics of gene transfer between species. Trends Genet. 7: 181-185.

Heinemann, J.A. & Sprague, G.F. 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. Nature 340: 205-209.

Hicks, R.J. *et al.* 1990. Review and evaluation of the effects of xenobiotic chemicals on microorganisms in soil. Adv. Appl. Microbiol. 35: 197-253.

Higgs, D.R. *et al.* 1990. A major positive regulatory region located far upstream of the human a-globin gene locus. Genes Dev. 4: 1588-1601.

Ho, M.W. 1997. *Genetic engineering: dream or nightmare?* Gateway Books, Bath, UK. (ISBN 1-85860-051-0).

Ho, M.W. 1998. Gene technology and gene ecology of infectious diseases. Microb. Ecol. Hlth. Dis. 10: 33-59.

Hofmann, C. & Strauss, M. 1998. Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. Gene Therapy 5: 531-536.

Houck, M.A. *et al.* 1991. Possible horizontal transfer of *Drosophila* genes by the mite *Proctolaelaps regalis*. Science 253: 1125-1129.

Hug, P. & Sleight, R.G. 1991. Liposomes for the transformation of eucaryotic cells. Biochim. Biophys. Acta 1097: 1-17.

Huxley, C. 1994. Mammalian artificial chromosomes: a new tool for gene therapy. Gene Therapy 1: 7-12.

Hyde, S. *et al.* 1993. Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy. Nature 362: 250-255**.**

Ikawa, Y. *et al.* 1974. An association between globin messenger RNA and 60S RNA derived from Friend leukemia virus. Proc. Natl. Acad. Sci. USA 71: 1154-1158.

Ikeno, M. *et al.* 1998. Construction of YAC-based mammalian artificial chromosomes. Nat. Biotechnol. 16: 431-439.

Inose, T. & Murata, K. 1995. Enhanced accumulation of toxic compound in yeast cells having high glycolytic activity: a case study on the safety of genetically engineered yeast. Int. J. Food Science Tech. 30: 141-146.

Isfort, R.J. *et al.* 1994. Retrovirus insertion into herpes viruses. TIM 2: 174-177.

Izant, J.G. & Weintraub, H. 1985. Constituitive and conditional suppression of exogenous and endogeneous genes by anti-sense RNA. Science 229: 345-352.

Jackson, D.A. *et al.* 1972. Biochemical method for inserting new genetic information into DNA of simian virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *E. coli*. Proc. Natl. Acad. Sci. USA 69: 2904-2909,

James, C. 1997. Global status of transgenic plants in 1997. ISAAA Briefs No. 5, ISAAA, Ithaca, New York.

Jarvik, T. & Lark, K.G. 1998. Characterization of Soymar1, a mariner element in soybean. Genetics 149: 1569-1574.

Jehle, J.A. *et al.* 1995. TC.14.7: a novel transposon found in *Cydia pomonella* granulosis virus. Virology 207: 369-379.

Jehle, J.A. *et al.* 1998. Horizontal escape of the novel Tc1-like lepidopteran transposon TCp3.2 into *Cydia pomonella* granulovirus. J Mol Evol 46: 215-224.

Johansen, S. & Vogt, V.M. 1994. An intron in the nuclear ribosomal DNA of *Didymium iridis* codes for a group I ribozyme and a novel ribozyme that cooperate in self-splicing. Cell 76: 725-734.

Kaprelyants, A.S. *et al.* 1993. Dormancy in nonsporulating bacteria. FEMS microbiol. Rev. 104: 271-286.

Kashani-Sabet, M. *et al.* 1992. Reversal of the malignant phenotype by an anti-Ras ribozyme. Antisense Res. Dev. 2: 3-15.

Kato, S. *et al.* 1986. Foreign DNA introduced by calcium phosphate is integrated into repetitive DNA elements of the mouse L genome. Mol. Cell Biol. 6: 1787-1795.

Keating, A. & Toneguzzo, F. 1990. Gene transfer by electroporation: a model for gene therapy. Prog. Clin. Biol. Res. 333: 491-498.

Kellog, S.T. *et al.* 1981. Plasmid assisted molecular breeding: new technique for enhanced biodegradation of resistant toxic chemicals. Science 214: 1133-1135.

Khanna, M. & Stozky, G. 1992. Transformation of *Bacillus subtilis* by DNA bound on montmorillonite and effect of DNase on the tranforming ability of bound DNA. Appl. Environ. Microbiol. 58: 1930-1939.

Kidambi, S.P. *et al.* 1994. Evidence for phage-mediated gene transfer among *Pseudo-monas aeruginosa* strains on the phylloplane. Appl. Environ. Microbiol. 60: 496-500.

Kidwell, M.G. 1993. Lateral transfer in natural populations of eukaryotes. Annu. Rev. Genet. 27: 235-256.

Kievits, T. *et al.* 1991. NASBA isothermal enzymatic *in vitro* nucleic acid amplification optimized for the diagnosis of HIV-1 infection. J. Virol. Methods 35: 273-286.

Kikuchi, Y. & Sasaki, N. 1991. Site-specific cleavage of natural RNA sequences by newly designed hairpin catalytic RNAs. Nucleic Acids Res. 19: 6751-6755.

Kipling, D. & Kearsey, S.E. 1990. Reversion of autonomously replicating sequence mutations in *Saccharomyces cerevisiae*: creation of a eucaryotic replication origin with procaryotic vector DNA. Mol. Cell. Biol. 10: 265-272.

Kitajima, I. *et al.* 1992a. Ablation of transplanted HTLV-I Tax-transformed tumors in mice by antisense inhibition of NF-kappa B. Science 258: 1792-1795.

Kitajima, I. *et al.* 1992b. Human T-cell leukemia virus type 1 tax transformation is associated with increased uptake of oligodeoxynucleotides *in vitro* and *in vivo*. J. Biol. Chem. 267: 25881-25888.

Koizumi, M. *et al.* 1992. Ribozymes designed to inhibit transformation of NIH 3T3 cells by the activated c-Ha-ras gene. Gene 117: 179-184.

Kokjohn, T.A. 1989. Transduction: mechanism and potential for gene transfer in the environment. Pp. 73-97 in: Levy, S.B. & Miller, R.V. (eds.) *Gene transfer in the environ-ment*. McGraw-Hill, New York.

Kolstø, A.-B. & Prydz, H. 1994. *Deteksjon av genspredning ved hjelp av PCR-teknikk*. Direktoratet for Naturforvaltning, Trondheim.

Kronenwett, R. & Haas, R. 1998. Antisense strategies for the treatment of hematologic-al malig-nancies and solid tumors. Ann. Hematol. 77: 1-12.

Kruger, K. *et al.* 1982. Self splicing RNA: auto excision and auto cyclization of the ribosomal RNA intervening sequence in *Tetrahymena thermophila*. Cell 31: 147-158.

Kruse, H. 1994. *Antimicrobial resistance - epidemiological aspects*. Dr. Scient. thesis, Norwegian College of Veterinary Medicine, Oslo (ISBN 82-7725-015-0).

Kruse, H. & Sørum, H. 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl. Environ. Microbiol. 60: 4015-4021.

Kruse, H. & Jansson, J. 1997. *The use of antibiotic resistance genes as marker genes in genetically modified organisms*. Norwegian Pollution Control Authority, Oslo (ISBN 82-7655-052-5.

Krysan, P.J. *et al.* 1989. Isolation of human sequences that replicate autonomously in human cells. Mol. Cell. Biol. 9: 1026-1033.

Kulka, M. *et al.* 1993. Antiviral effect of oligo (nucleoside methylphosphonates) comple-mentary to the herpes simplex virus type 1 immediate early mRNAs 4 and 5. Antiviral Res. 20: 115-130.

Kusano, K. *et al.* 1997. A new type of illegitimate recombination is dependent on restriction and homologous interaction. J. Bacterial 179: 5380-5390.

Lamb, B.T. *et al.* 1993. Introduction and expression of the 400 kilobase precursor amyloid protein gene in transgenic mice. Nat. Genet. 5: 22-29.

Lambowitz, A.M. 1989. Infectious introns. Cell 56: 323-326.

Lambowitz, A.M. & Belfort, M. 1993. Introns as mobile genetic elements. Annu. Rev. Biochem. 62: 587-622.

Landman, O.E. 1991. The inheritance of acquired characteristics. Annu. Rev. Genet. 25: 1-20.

Latorre, J. *et al.* 1984. Microbial metabolism of chloroanilines: enhanced evolution by natural genetic exchange. Arch. Microbiol. 140: 159-165,

Lazowska, J. *et al.* 1994. Homing of a group II intron in yeast mitochondrial DNA is accompanied by unidirectional co-conversion of upstream-located markers. EMBO J. 13: 4963-5972.

Leaning, J. 1994. Questions coming late in the day. Environ. Impact Assess. Rev. 14: 321-326.

Lee, H. et al. 1994. Bacterial mutagenicity, metabolism, and DNA adduct formation by binary mixtures of benzo(a)pyrene and 1-nitropyrene. Environ. Mol. Mutagen. 24: 229-234.

Leeton, P.R.J. & Smyth, D.R. 1993. An abundant LINE-like element amplified in the genome of *Lilium speciosum*. Mol. Gen. Genet. 237: 97-104.

Leff, L.G. *et al.* 1992. Information spiraling: movement of bacteria and their genes in streams. Microb. Ecol. 24: 11-24.

Leung, S.S. & Romans, P. 1998. Excisions of the Ikara1 transposon in an *Anopheles gambiae* cell line. Insect Mol. Biol. 7: 241-248.

Levine, F. & Friedman, T. 1993. Gene therapy. AJDC 147: 1167-1174.

Levy, S.B. & Miller, R.V. 1989. *Gene transfer in the environment*. McGraw-Hill, New York.

Liang, P. *et al.* 1993. Distribution and cloning of eucaryotic mRNAs by means of differential display: refinements and optimalization. Nucl. Acids Res. 21: 3269-3275.

Lo, K.M. *et al.* 1992. Inhibition of replication of HIV-1 by retroviral vectors expressing tat-antisense and anti-tat ribozyme RNA. Virology 190: 176-183.

Loke, S.L. *et al.* 1989. Characterization of oligonucleotide transport into living cells. Proc. Natl. Acad. Sci. USA 86: 3474-3478.

Lorenz, M.G. 1992. Gene transfer via transformation in soil/sediment environments. Pp. 95-101 in: Gauthier, M.J. (ed.) *Gene transfers and environment*. Springer Verlag KG, Berlin.

Lorenz, M.G. & Wackernagel, W. 1991. High frequency of natural genetic transforma-tion of *Pseudomonas stutzeri* in soil extract supplemented with a carbon/energy and phosphorous source. Appl. Environ. Microbiol. 57: 1246-1251.

Lorenz, M.G. & Wackernagel, W. 1992. Stimulation of natural genetic transformation of *Pseudomonas stutzeri* in extracts of various soils by nitrogen or phosphorous limitation and influence of temperature and pH. Microb. Releases 1: 173-176.

Lorenz, M.G. & Wackernagel, W. 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol. Rev. 58: 563-602.

Lorenz, M.G. *et al*. 1991. Release of transforning plasmid and chromosomal DNA from two cultured soil bacteria. Arch. Microbiol. 156: 319-326.

Lundgren, B. & DePierre, J.W. 1990. The metabolism of xenobiotics and its relation-ship to toxicity/genotoxicity: studies with human lymphocytes. Acta Physiol. Scand. Suppl. 592: 49-59.

Luo, G. *et al*. 1998. Chromosomal transposition of a Tc1/mariner-like element in mouse embryonic stem cells. Proc. Natl. Acad. Sci. USA 95: 10769-10773.

Lyerly, K.H. & DiMaio, J.M. 1993. Gene delivery systems in surgery. Arch. Surg. 128: 1197-1206.

Mattick, J.S. 1994. Introns: evolution and function. Curr. Biol. Gen. Devel. 4: 823-831.

Maturin, L. & Curtiss, R. 1977. Degradation of DNA by nucleases in intestinal tract of rats. Science 196: 216-218.

Maynard Smith, J. *et al*. 1991. Localized sex in bacteria. Nature 349: 29-31.

Mazodier, P. & Davies, J. 1991. Gene transfer between distantly related bacteria. Annu. Rev. Genet. 25: 147-171.

McAllan, A.B. 1982. The fate of nucleic acids in ruminants. Prog. Nutr. Soc. 41: 309-317.

McClure, M.A. 1991. Evolution of retroposons by acquisition or deletion of retrovirus-like genes. Mol. Biol. Evol. 8: 835-856.

McMichael, A.J. 1993. *Planetary overload: global environmental change and the health of the human species*. Cambridge University Press, Cambridge.

McNally, R. 1994. Genetic madness. The European rabies eradication programme. The Ecologist 24: 207-212.

McNaughton, J.C. *et al*. 1997. The evolution of an intron: analysis of a long, deletion-prone intron in the human dystrophin gene. Genomics 40: 294-304.

Mejean, V. & Claverys. J.-P. 1988. Polarity of DNA entry in transformation of *Strepto-coccus pneumoniae*. Mo. Gen. Genet. 222: 851-856, 444-448.

Mejean, V. & Claverys. J.-P. 1993. DNA processing during entry in transformation of *Streptococcus pneumoniae*. J. Biol. Chem. 268: 5594-5599.

Michel, F. & Westhof, E. 1990. Modelling of the three-dimensional architecture of group I catalytic introns based on comparative sequence analysis. J. Mol. Biol. 216: 585-610.

Mikkelsen, T.R. *et al*. 1996. The risk of crop transgene spread. Nature 380: 31.

Miller, D.W. & Miller, L.K. 1982. A virus with an insertion of a copia-like element. Nature 299: 562-564.

Mizuno, T. *et al*. 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (mic RNA). Proc. Natl. Acad. Sci. USA 81: 1966-1970.

Montgomery, D.L. *et al*. 1993. Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. DNA Cell Biol. 12: 777-783.

Morell, V. 1993. Dino DNA: the hunt and the hype. Science 261: 160-162.

Morgan, R.A. & Anderson, W.F. 1993. Human gene therapy. Annu. Rev. Biochem. 62: 191-217.

Moxham, C.M. *et al*. 1993. Induction of Ga_{12}-specific antisense RNA *in vivo* inhibits neonatal growth. Science 260: 991-995.

Nabel, G.J. *et al*. 1992. Gene transfer *in vivo* with DNA-liposome complexes: lack of autoimmunity and gonadal localization. Hum. Gene Ther. 3: 399-410.

Needham, D. *et al*. 1992. Repulsive interactions and mechanical stability of polymer-grafted lipid membranes. Biochim. Biophys. Acta 1108: 40-48.

Nesburn, A.B. *et al*. 1994. Vaccine therapy for ocular *Herpes simplex* virus infection: periocular vaccination reduces spontaneous ocular HSV type 1 shedding in latently infected rabbits. J. Virol. 68: 5084-5092.

Newman, A. 1995. Mercury increases DNA damage from radiation (commentary). Environ. Sci. Technol. 29: 18A.

Nielsen, K.M. *et al*. 1998. Horizontal gene transfer from transgenic plants to terrestrial bacteria – a rare event? FEMS Microbiological Reviews 22: 79-103.

Nikolich, N. *et al*. 1994. Evidence for natural horizontal transfer of tet Q between bacteria that normally colonize humans and bacteria that normally colonize livestock. Appl. Environ. Microbiol. 60: 3255-3260.

Nordlee, J.A. *et al*. 1996. Identification of a Brazil-nut allergen in transgenic soybeans. New Engl. J. Med. 14: 688-728.

Novitzky, J.A. 1986. Degradation of dead microbial biomass in a marine sediment. Appl. Environ. Microbiol. 52: 504-509.

Ogram, O.V. *et al*. 1994. Effects of DNA polymer length on its adsorption to soils. Appl. Environ. Microbiol. 60: 393-396.

Ogunseitan, O.A. *et al*. 1992. Application of DNA probes to analysis of bacteriophage distribution patterns in the environment. Appl. Environ. Microbiol. 58: 2046-2052.

Outwater, J.L. *et al*. 1997. Dairy products and breast cancer: the IGF-1, estrogen and bGH hypothesis. Med. Hypotheses 48: 453-61.

Page, W.J. 1985. Genetic transformation of molybdenum-starved *Azobacter vinelandii*: increased transfornation frequency and recipient range. Can. J. Microbiol. 31: 659-662.

Page, W.J. & Doran, J.L. 1981. Recovery of competence in calcium-limited *Azobacter vinelandii*. J. Bacteriol. 146: 33-40.

Page, W.J. & Sadoff, H.L. 1976. Physiological factors affecting transformation of *Azobacter vinelandii*. J. Bacteriol. 125: 1080-1087.

Paul, J.H. & David, A.W. 1989. Production of extracellular nucleic acids by genetically altered bacteria in aquatic-environment microcosms. Appl. Environ. Microbiol. 55: 1865-1867.

Paul, J.H. *et al*. 1991. Gene transfer in marine water column and sediment microcosms by natural plasmid transformation. Appl. Environ. Microbiol. 57: 1509-1515.

Perrouault, L. *et al*. 1990. Sequence-specific artificial photoinduced endonucleases based on triple helix-forming oligonucleoties. Nature 344: 358-360.

Powers, D.A. *et al*. 1991. Genetic mechanisms for adapting to a changing environment. Annu Rev. Genet. 25: 629-659.

Pääbo, S. *et al*. 1988. Mitochondrial DNA sequences from a 7000 year old brain. Nucleic Acids Res. 16: 9777-9787.

Raviprakash, K. *et al*. 1995. Inhibition of Dengue virus by novel, modified antisense oligonucleotides. J. Virol. 69: 69-74.

Raz, *et al*. 1993. Systematic immunological effects of cytokine genes injected into skeletal muscle. Proc. Natl. Acad. Sci. USA 90: 4523-4527.

Recorbet, G. *et al*. 1993. Kinetics of persistence of chromosomal DNA from genetically engineered *Escherichia coli* introduced into soil. Appl. Environ. Microbiol. 59: 4289-4294.

Reddy, S.A. & Thomas, T.L. 1996. Expression of a cyanobacterial delta 6-desaturase gene results in gamma-linolenic acid production in transgenic plants. Nature Biotechnol. 14: 639-42.

Rekvig, O.P. *et al*. 1992. Antibodies to eukaryotic, including autologous, native DNA are produced during BK virus infection, but not after immunization with non-infectious BKV DNA. Scand. J. Immunol. 36: 487-495.

Rissler, J. & Mellon, M. 1996. *The ecological risks of engineered crops*. MIT Press, Cambridge, Massachusetts (ISBN 0-262-68085-8).

Romanowski, G. *et al*. 1991. Adsorption of plasmid DNA to mineral surfaces and protection against DNase I. Appl. Environ. Microbiol. 57: 1057-1061.

Romanowski, G. *et al*. 1992. Persistence of free plasmid DNA in soil monitored by various methods, including a transformation assay. Appl. Environ. Microbiol. 58: 3012-3019.

Romanowski, G. *et al*. 1993. Use of a polymerase chain reaction and electroporation of *Escherichia coli* to monitor the persistence of extracellular plasmid DNA introduced into natural soils. Appl. Environ. Microbiol. 59: 3438-3446.

Romanowski, G. *et al*. 1993. Plasmid DNA in a groundwater aquifer microcosm - adsorption, DNase resistance and natural genetic transformation of *Bacillus subtilus*. Mol. Ecol. 2: 171-181.

Rossi, J.J. 1998. Ribozymes to the rescue: repairing genetically defective mRNAs. Trends Genet. 14: 295-298.

Rowji, P. 1989. Genetic transformation in encapsulated clinical isolates of *Haemophilus influenzae* type b. J. Gen Microbiol. 135: 2775-2782.

Saison-Behmoaras, T. *et al*. 1991. Short modified anti-sense nucleotides directed against Ha-ras point mutation induce selective cleavage of the mRNA and inhibit T24 cell proliferation. EMBO J. 10: 1111-1118.

Saldanha, R. *et al*. 1993. Group I and group II introns. FASEB J. 7: 15-24.

Scanlon, K.J. *et al*. 1991. Ribozyme-mediated cleavage of c-fos mRNA reduces expres-sion of DNA synthesis enzymes and methallothionein. Proc. Natl. Acad. Sci. USA 88: 10591-10595.

Schlimme, W. *et al*. 1997. Gene transfer between bacteria within digestive vacuoles of protozoa. FEMS Microbiol Ecol 23: 239-247.

Schouten, G.J. *et al*. 1998. Transposon Tc1 of the nematode *Caenorhabditis elegans* jumps in human cells. Nucleic Acids Res 26: 3013-3017.

Schrempf, H. 1985. Genetic instability: amplification, deletion and rearrangements within Streptomyces DNA. Pp. 436-440 in: Leive, L. (ed.) *Microbiology-1985*.

Schubbert, R. *et al*. 1994. Ingested foreign (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. Mol. Gen. Genet. 242: 495-504.

Schubbert, R. *et al.* 1997. Foreign (m13) DNA ingested by mice reaches peripheral leukocytes spleen and liver via intestinal wall mucosa and can be covalently linked to mouse DNA. Proc. Natl. Acad. Sci. USA 94: 961-966.

Shima, N. & Ichikawa, S. 1994. Synergisms detected among methylmethanesulfonate, ethyl methanesulfonate and X-rays in inducing somatic mutations in the stamen hairs of Tradescantia clone BNL 4430. Environ. Exp. Botany 34: 393-408.

Sidransky, H. *et al.* 1994. Studies with 1,1´-ethylidenebis (tryptophan), a contaminant associated with L-tryptophan implicated in the eosinophilia-myalgia syndrome. Toxicol. Appl. Pharmacol. 126: 108-13.

Sioud, M. *et al.* 1992. Preformed ribozyme destroys tumour necrosis factor mRNA in human cells. J. Mol. Biol. 223: 831-835.

Smets, B.F. *et al.* 1993. The specific growth rate of *Pseudomonas putida* PAW1 influences the conjugal transfer rate of the TOL plasmid. Appl. Environ. Microbiol. 59: 3430-3437.

Smital, T. & Kurelec, B. 1998. The chemosensitizers of multixenobiotic resistance mechanism in aquatic invertebrates: a new class of pollutants. Mutat. Res. 399: 43-53.

Smith, H.O. *et al.* 1981. Genetic transformation. Annu. Rev. Biochem. 50: 41-68.

Smith, J.G. *et al.* 1993. Liposomes as agents of DNA transfer. Biochim. Biophys. Acta 1154: 327-340.

Smith, K.A. *et al.* 1990. Distinctive chromosomal structures are formed very early in the amplification of the CAD genes in Syrian hamster cells. Cell 63: 1219-1227.

Smith, M.W. *et al.* 1992. Evolution by acquisition: the case for horizontal transfers. Trends Biochem. Sci. 17: 489-493.

Sparling, P.F. 1966. Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. J. Bacteriol. 92: 1364-1371.

Sprague, G.F. jr. 1991. Genetic exchange between kingdoms. Curr. Opin. Genet. Dev. 1: 530-533.

Spring, S. *et al.* 1992. Phylogenetic diversity and identification of nonculturable magneto-tactic bacteria. Syst. Appl. Microbiol. 15: 116-122.

Stachel, S.E. & Zambryski, P.C. 1989. Generic trans-kingdom sex? Nature 340: 190-191.

Steffan, R.J. *et al.* 1988. Recovery of DNA from soils and sediments. Appl. Environ. Microbiol. 54: 2908-2915.

Stein, C.A. 1992. Anti-sense oligodeoxynucleotides-promises and pitfalls. Leukemia 6: 967-974.

Stein, C.A. *et al*. 1993. Dynamics of the internalization of phosphodiester oligodeoxy-nucleotides in HL60 cells. Biochemistry 32: 4855-4861.

Stein, D.C. 1991. Transformation of *Neisseria gonorrhoeae*: physical requirements of the transforming DNA. Can. J. Microbiol. 37: 345-349.

Stein, J.E. *et al*. 1990. Overview of studies on liver carcinogenesis in English sole from Puget Sound; evidence for a xenobiotic chemical etiology. II. Biochemical studies. Sci. Total Environ. 94: 51-69.

Steinmetz, R. *et al*. 1998. The xenoestrogen biphenol A induces growth, differentiation, and c-fos gene expression in the female reproductive tract. Endocrinology 139: 2741-2747.

Stephens, R.S. *et al*. 1998. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. Science 282: 754-759,.

Stewart, M.J. *et al*. 1992. Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. Biochim. Biophys. Acta 1113: 201-227.

Stotzky, G. *et al*. 1993. Selected methods for the detection and assessment of ecological effects resulting from the release of genetically engineered microorganisms to the terrestrial environment. Adv. Appl. Microbiol. 38: 1-98.

Stribling, R. *et al*. 1992. Aerosol gene delivery *in vivo*. Proc. Natl. Acad. Sci. USA 89: 11277-11281.

Sun, R. *et al*. 1993. Pp. 279-284 in: Turz, T. *et al*. (eds.) *The Epstein-Barr virus and associated diseases*. Colloque INSERM/John Libby Eurotext.

Syvänen, M. 1984. Conserved regions in mammalian b-globulins: could they arise by cross-species gene exchange. J. Theor. Biol. 107: 685-696.

Syvänen, M. 1985. Cross-species gene transfer: implications for a new theory of evolution. J. Theor. Biol. 112: 333-343.

Syvänen, M. 1987. Cross-species gene transfer: a major factor in evolution? Trends Genet. 2: 63-66.

Syvänen, M. 1987. Molecular clocks and evolutionary relationships: possible distortion due to horizontal gene flow? J. Mol. Evol. 26: 16-23.

Syvänen, M. 1994. Horizontal gene transfer: evidence and possible consequences. Annu. Rev. Genet. 28: 237-261.

Tang, D. *et al*. 1992. Genetic immunization is a simple method for eliciting an immune response. Nature 356: 152-154.

Thakur, M.S. *et al*. 1991. An environmental assessment of biotechnological processes. Adv. Appl. Microbiol. 36: 67-86.

Thiem, S.M. *et al*. 1994. Use of molecular techniques to evaluate the survival of a micro-organism injected into an aquifer. Appl. Environ. Microbiol. 60: 1059-1067.

Thomson, C.J. *et al*. 1994. TEM-1 plasmids in the community. Lancet 343: 921.

Timmis, J.N. & Scott, N.S. 1984. Promiscuous DNA: sequence homologies between DNA of separate organelles. Trends Biochem. Sci. 9: 271-273.

Toh, H. *et al*. 1985. Close structural resemblance between putative polymerase of a *Drosophila* transposable genetic element 17.6 and the pol gene product of Moloney murine leukemia virus. EMBO J. 4: 1267-1272.

Tomita, N. *et al*. 1992. Gene transfer *in vivo* with DNA-liposome complexes: safety and acute toxicity in mice. Hum. Gene Ther. 3: 267-275.

Torsvik, V.L. & Goksøyr, J. 1978. Determination of bacterial DNA in soil. Soil Biol. Biochem. 10: 7-12.

Traavik, T. 1979. *Arboviruses in Norway*. Dr. philos. thesis. University of Tromsø, Norway.

Trevors, J.T. *et al*. 1987. Gene transfer among bacteria in soil and aquatic environments: a review. Can. J. Microbiol. 33: 191-198,

Tsai, Y.-L. *et al*. 1995. Analysis of viral RNA persistence in seawater by reverse transcriptase-PCR. Appl. Environ. Microbiol. 61: 363-366.

Turk, V. *et al*. 1992. Release of bacterial DNA by marine nanoflagellates, an intermediate step in phosphorous regeneration. Appl. Environ. Microbiol. 58: 3744-3750.

Tyler, C.R. *et al*. 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit. Rev. Toxicol. 28: 319-361.

Ulmer, J.B. *et al*. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259: 1745-1749.

U.S. Environmental Protection Agency. 1994a. *Health assessment document for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds*. Government Print-ing Office, Wash-ington D.C.

U.S. Environmental Protection Agency. 1994b. *Estimating exposure to dioxin-like compounds, vol. I: Executive summary*. Government Printing Office, Washington D.C.

U.S. Environmental Protection Agency. 1994c. *Estimating exposure to dioxin-like compounds, vol. II: Properties, sources, occurrence and background exposures*. Govern-ment Printing Office, Washington D.C.

Vader, A. *et al.* 1994. Nuclear introns from *Physarum flavicomum* contain insertion elements that may explain how mobile group I introns gained their open reading frames. Nucleic Acids Res. 22: 4553-4559.

Vahlsing, H.L. *et al.* 1994. Immunization with plasmid DNA using a pneumatic gun. J. Immunol. Meth. 175: 11-22.

Violand, B.N. *et al.* 1994. Isolation of *Escherichia coli* synthesized recombinant eukaryotic proteins that contain epsilon-N-acetyllysine. Protein Sci. 3: 1089-97.

Vlassov, V.V. *et al.* 1994. Transport of oligonucleotides across natural and model memb-ranes. Biochim. Biophys. Acta 1197: 95-108.

Vogel, J.C. 1993. Keratinocyte gene therapy. Arch. Dermatol. 129: 1478-1483.

Walsh, C.E. *et al.* 1993. Gene therapy for human hemoglobinopathies. Proc. Soc. Exp. Biol. Med. 204: 289-300.

Wang, B. *et al.* 1993. Gene inoculation generates immune responses against human immunodefi-ciency virus type 1. Proc. Natl. Acad. Sci. USA 90: 4156-4160.

Wang, C.Y. & Huang, L. 1989. Highly efficient DNA delivery mediated by pH sensitive immu-noliposomes. Biochemistry 28: 9508-9514.

Whitton, J.L. 1994. Antisense treatment of viral infection. Adv. Virus Res. 44: 267-303.

Wickström, E. *et al.* 1992. Downregulation of c-MYC antigen expression in lymphocytes of Emu-c-myc transgenic mice treated with anti-c-myc DNA methyl-phosphonates. Cancer Res. 52: 6741-6745.

Widmer, F. *et al.* 1997. Quantification of transgenic marker gene persistence in the field. Mol. Ecol. 6: 1-7.

Wilhelmson, A. *et al.* 1990. The specific DNA binding activity of the dioxin receptor is modulated by the 90 kd heat shock protein. EMBO J. 9: 69-76.

Williams, D.E. *et al.* 1998. Xenobiotics and xenoestrogens in fish: modulation of cyto-chrome P450 and carcinogenesis. Mutat. Res. 399: 179-192.

Williams, N. 1998. Agricultural biotech faces backlash in Europe. Science 281: 768-71.

Williams, R.S. *et al*. 1991. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc. Natl. Acad. Sci. USA 88: 2726-2730.

Wilson, J.M. *et al*. 1992. Hepatocyte-directed gene transfer *in vivo* leads to transient improvement of hypercholesterolemia in low density lipoprotein-deficient rabbits. J. Biol. Chem. 267: 963-967.

Wirgin, I. & Waldman, J.R. 1998. Altered gene expression and genetic damage in North American fish populations. Mutat Res 399: 193-219.

Wivel, N.A. & Wilson, J.M. 1998. Methods of gene delivery. Hematol. Oncol. Clin. North Am. 12: 483-501.

Wolff, J.A. *et al*. 1990. Direct gene transfer into mouse muscle *in vivo*. Science 247: 1465-1468.

Woolf, T.M. 1998. Therapeutic repair of mutated nucleic acid sequences. Nature Biotechnol. 16: 341-344.

Wöstemayer, J. *et al*. 1997. Horizontal gene transfer in the rhizophere: a curiosuty or a driving force in evolution? Adv. Bot. Res. Incorp. Adv. Plant Pathol. 24: 399-429.

Xiang, Z.Q. *et al*., 1994. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. Virology 199: 132-140.

Xing, Z. & Whitton, J.L. 1993. An anti-LCMV ribozyme expressed in tissue culture cells diminishes viral RNA levels, and leads to a reduction in infectious virus yield. J. Virol. 66: 1361-1369.

Yakubov, L.A. *et al*. 1989. Mechanism of oligonucleotide uptake by cells: involvement of specific receptors? Proc. Natl. Acad. Sci. USA 86: 8454-8458.

Yother, J. *et al*. 1986. Transformation of encapsulated *Streptococcus pneumoniae*. J. Bacteriol. 168: 1463-1465.

Yu, M. *et al*. 1994. Progress towards gene therapy for HIV infection. Gene Therapy 1: 13-26.

Zacharewski, T. 1998. Identification and assessment of endocrine disrupters: limitations of *in vivo* and *in vitro* assays. Enviro. Health Perspect. 106 Suppl 2: 577-582.

Zambryski, P.C. *et al*. 1989. Transfer and function of T-DNA genes from Agrarium Ti and Ri plasmids. Cell 56: 193-201.

Zhou, Y. *et al*. 1993. Transfer of neurotoxigenicity from *Clostridium butyricum* to a non-toxigenic *Clostridium botulinum* type E-like strain. Appl. Environ. Microbiol. 59: 3825-3831.

Zhu, N. *et al*. 1993. Systemic gene expression after intravenous DNA delivery into adult mice. Science 261: 209-211.

Zuchman-Rossi, J. *et al*. 1998. Chromosome translocation based on illegitimate recombination in human tumors. Proc. Natl. Acad. Sci. USA 95: 11786-11791.

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Corrections in the references:

Abbot, A. 1997. Austrian gene food petition puts pressure on European partners. Nature 386: 745

Alvarez, A.J. *et al.* 1996. Stability of manipulated plasmid DNA in aquatic environments. Environ. Toxicol. Water Quality 11: 129-135

Australian Genethics Network 1994. *The troubled helix. Vol. 3* (ISBN 0-85802-115-3 08502-095-5).

Benton, P.A. & Kennedy, R.C. 1998. DNA vaccine strategies for the treatment of cancer. Curr. Top. Microbiol. 226: 1-20.

Bergh, O. *et al.* 1989. High abundance of viruses found in aquatic environments. Nature 340: 467-468.

Blum, S.A.E. *et al.* 1997. Persistence of DNA in natural soils: Adsorption to particulate material provides protection against nucleolytic degradation. Pp. 147-155 in: Schmidt, E. R. & Hankeln, T. (eds.) *Transgenic organisms and biosafety*. Springer Verlag, Berlin.

Cameron, J. & Abouchar, J. 1991. The precautionary principle. Boston College international and comparative law review 1991. XIV: 1-28.

Capecchi, M.R. 1994. Targeted gene replacement. Sci. Am. 270: 352-359.

Coghlan, A. 1994. Will the scorpion gene run wild? New Scientist 25 June 1994, pp. 14-15.

Cohen, J. 1994. Vaccines get a new twist. Science 264: 503-505.

Colborn, T. 1995a. Environmental estrogens: health implications for humans and wildlife. Environ. Health Perspect. 103, suppl. 7: 135-136.

Colborn, T. 1995b. Pesticides – how research has succeeded and failed to translate science into policy: endocrine effecxts on wildlife. Environ. Health Perspect. 103, suppl. 6: 81-85.

Colborn, T. *et al.* 1998. Environmental neurotoxic effects: the search for new protocols in functional teratology. Toxicol. Ind. Health 14: 9-23.

Daane, L.L. *et al.* 1996. Influence of earthworm activity on gene transfer from *Pseudo-monas fluorescens* to indigenous soil bacteria. Appl. Environ. Microbiol. 62: 515-521.

Davidson, B.L. *et al.* 1993. A model system for *in vivo* gene transfer into the central nervous system using an adenovirus vector. Nature Genet. 3: 219-223.

Davies, J. 1994. Inactivation of antibiotics and the dissemination of resistance genes. Science 264: 375-382.

Davis, H.L. *et al.* 1993. DNA-based immunization induces continuous secretion of hepatitis B surface antigen and high levels of circulating antibodies. Hum. Mol. Gen. 2: 1847-1851

Doolittle, W.F. 1998. You are what you eat: a gene transfer ratchet could account for bacterial genes in eucaryotic nuclear genomes. Trends Genet. 14: 307-11.

Dröge, M. *et al.* 1998. Horizontal gene transfer as a biosafety issue: a natural phenomenon of public concern. J. Biotechnol. 64: 75-90.

Einvik, C. *et al.* 1998. Group I twintrons: genetic elements in myxomycete and schizopyrenid amoebofgallate ribosomal DNAs. J. Biotechnol. 64: 63-74.

Finnegan, D.J. 1989. Eukaryotic transposable elements and genome evolution. Trends Genet. 5: 103-107.

Gray, LE. *et al.* 1997. Endocrine screening methods workshop report: detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. Reprod. Toxicol. 11: ,719-750

Gruzza, M. *et al.* 1993. Gene transfer from engineered *Lactobacillus lactis* strains to *Enterococcus faecalis* in the digestive tract of gnotobiotic mice. Microb. Releases 2: 121-125.

Hamlin, J.L 1992. Mammalian origins of replication. BioEssays 14: 651-659.

Ho, M.W. 1998. *Genetic engineering: dream or nightmare?* Gateway Books, Bath, UK. (ISBN 1-85860-051-0).

Ho, M.W. *et al.* **1998.** Gene technology and gene ecology of infectious diseases. Microb. Ecol. Hlth. Dis. 10: 33-59.

Ivics, Z.*et al.* 1996. Identification of functional domains and evolution of Tc1-like transposable elements. Proc. Natl. Acad. Sci. USA 93: 5008-5013

Ivics, Z. *et al.* 1997. Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposable cells. Cell 91: 501-510.

Johansen, S. *et al*. 1996. Insertion elements in ribosomal DNA. In: Zimmermann, R.A. and Dahlberg, A.E. (Eds.), Ribosomal RNA:Structure, Evolution, Processing, and Function in Protein Biosynthesis. CRC Press, Boca Raton, FL, pp. 89-108.

Johansen, S. *et al.* 1997. Group I introns in biotechnology: prospects of application of ribozymes and rare-cutting homing nucleases. Biotechnol. Ann. Rev. 3: 111-150.

Kilby, N.J. *et al.* 1993. Site-specific recombinases: tools for genome engineering. TIG 9: 413-420.

Kouskoff, V. *et al*. 1993. A vector driving the expression of foreign cDNAs in the MHC class II-positive cells of transgenic mice. J. Immunol. Meth. 166: 287-291.

Letvin, N.L.1998. Progress in the development of an HIV-1 vaccine. Science 280: 1875-1880.

Mahsod, E. 1998. Call for UK genetics food watchdog. Nature 595: 5.

Mercenier & Chassy, 1988. Strategies for development of bacterial transformation systems. Biochimie 70: 503-517.

Michaels, M.G. 1997. Infectious concerns of cross-species transplantation: xenozoo-noses. World J. Surg. 21: 968-974.

Milot, E. *et al*. 1994. Association of a host DNA structure with retroviral integration sites in chromosomal DNA. Virology 201: 408-412.

Ogram, A. *et al*. 1988. DNA adsorption to soils and sediments. Environ. Sci. Technol. 22: 982-984.

Raz, E. *et al*. 1993. Systematic immunological effects of cytokine genes injected into skeletal muscle. Proc. Natl. Acad. Sci. USA 90: 4523-4527.

Renneisen, K. *et al*. 1990. Inhibition of expression of human immunodeficiency virus 1 in vitro by antibody-targeted liposomes containing antisense RNA to the env region. J. Biol. Chem. 265: 16337-16342.

Resnikoff, J.R. & Conrad, D.J.1998. Recent advances in understanding and treatment of cystic fibrosis. Curr. Opin. Pulm. Med. 4: 130-134.

Romanowski, G. *et al*. 1993a. Use of a polymerase chain reaction and electroporation of *Escherichia coli* to monitor the persistence of extracellular plasmid DNA introduced into natural soils. Appl. Environ. Microbiol. 59: 3438-3446

Romanowski, G. *et al*. 1993b. Plasmid DNA in a groundwater aquifer microcosm - adsorption, DNase resistance and natural genetic transformation of *Bacillus subtilus*. Mol. Ecol. 2: 171-181.

Rowji, P. *et al.* **1989.** Genetic transformation in encapsulated clinical isolates of *Haemophilus influenzae* type b. J. Gen Microbiol. 135: 2775-2782.

Rural Advancement Foundation International, 1998. The terminator technology. New genetic technology aims to prevent farmers from saving seed. RAFI Communique, March/April 1998, Winnipeg, Canada.

Simons, R.W. 1988. Naturally occurring antisense RNA control - a brief review. Gene 72: 35-44

Smith, M.W. & Doolittle, R.F. 1992. Anomalous phylogeny involving the enzyme glucose-6-phosphate isomerase. J. Mol. Evol. 34: 544-545

Sorensen, S.J. 1993. Transfer of plasmid RP4 from *Escherichia coli* K-12 to indigenous bacteria of seawater. Microb. Releases 2: 135-140

Stevens, S.W. & Griffith, J.D. 1994. Human immunodeficiency virus type 1 may prefer-entially integrate into chromatin occupied by L1Hs repetitive elements. Proc. Natl. Acad. Sci. USA 91: 5557-5561

Stewart, G.J. *et al*. 1991. Binding of exogenous DNA to marine sediments and the effect of DNA/sediment binding on natural transformation of *Pseudomonas stutzeri* strain ZoBell in sediment columns. FEMS Microbiol. Ecol. 85: 1-8

Stotzky, G. 1989. Gene transfer among bacteria in soil. In: Gene Transfer in the Environment (Levy, S.B. and Miller, S.V., Eds.). pp 165-222. McGraw-Hill, New York.

Sun, T.-Q. *et al.* 1994. Human artificial episomal chromosomes for cloning large DNA frag-ments in human cells. Nature Genetics 8: 33-41.

Syvänen, M. 1987a. Cross-species gene transfer: a major factor in evolution? Trends Genet. 2: 63-66

Syvänen, M. 1987b. Molecular clocks and evolutionary relationships: possible distortion due to horizontal gene flow? J. Mol. Evol. 26: 16-23.

Taylor, R.N. *et al*. 1992. Chimeric DNA-RNA hammerhead ribozymes have enhanced *in vitro* catalytic efficiency and increased stability *in vivo*. Nucleic Acids Res. 20: 4559-4565

Wilson, M. & Lindow, S.E. 1993. Release of recombinant microorganisms. Annu. Rev. Microbiol. 47: 913-944

Wuetrich, B. 1994. Will altered plants breed deadlier diseases? New Scientist 2 April 1994, p. 15.

Zhao, J.J. & Lemke, G. 1998. Rules for ribozymes. Mol. Cell. Neurosci. 11: 92-97.

Glossary

Allele: A site on the chromosome which is the location for one or more specific genes. Each chromosome contains an allele. Diploid organisms therefore have two alleles.

Allogenic: Used about transplantation; transfer of tissue between individuals within the same species.

Anastomosis: Free connection between two canals, such as between two blood vessels. Also used about nerve fibres, etc.

Antagonism: Conflict, hostility. Used when two chemical substances have the opposite effect, such as poison and antidote.

Antibody: A protein which is produced when the immune system recognises a foreign intruder (molecule, virus, micro-organism) and which has the task of rendering the intruder harmless.

Antigen: A molecule or substance (generally of proteinic nature) which will stimulate the immune system to produce antibodies when introduced into a vertebrate.

Anti-sense: A nucleotide sequence (RNA or DNA) which is complementary to a given mRNA. The sequence in mRNA is considered as sense because it can be directly read as a protein.

Arbovirus: Acronym derived from *ar*thropd-*bo*rne *virus*, i.e. a virus which is transferred with the aid of arthropods, usually blood-sucking mosquitoes, mites or midges.

Arthropods: Articulated animals.

Autologous: Used in connection with transplantation of an individual's own tissue.

Bacteriophage: Virus which attacks and multiplies within bacteria cells.

Baculovirus: Derived from *baculum* = rod, which alludes to the appearance of the virus particles in the electron microscope. The *Baculoviridae* family is composed of structurally and genetically complex viruses which have insects as hosts, and have nothing in common with vertebrate viruses.

Base pair (bp): Two complementary bases which, with the help of hydrogen bonds, are capable of pairing themselves with one another from a position in their own opposing nucleic acid strand. Adenine pairs with thymine in DNA and uracil in RNA. Guanine pairs with cytosine.

Bases: Purine molecules (double rings which contain nitrogen) and pyrimidine molecules (single rings which contain nitrogen) that are present in DNA and RNA. They include adanine, guanine, thymidine, cytosine and uracil.

Biosphere: The area on and around the Earth where life is found. Includes the sea, the land and the lower part of the atmosphere.

Centromere: The area of a chromosome which is important for correct segregation during cell division. The sister chromatids attach together here following DNA replication.

Chlamydia: Bacteria with a strictly intracellular multiplication cycle. Cause diseases such as psittacosis, trachoma and lymphogranulorna venereum.

Chromosome: Carrier of hereditary characteristics, i.e. the genes. Each chromosome may contain up to a couple of thousand genes. Man has 46 chromosomes, 22 pairs and also the sexual chromosomes X/Y in males and X/X in females.

Clone: A group of molecules, cells or organisms all of which are genetically identical and are descended from a common ancestral molecule, cell or organism.

Codon: A group of three nucleotides that, together, code for one amino acid in the poly-peptide chain for which the entire gene codes.

Competence: In the present context, the ability of a cell to take up naked DNA.

Complementary DNA (cDNA): DNA which is produced by reverse transcription of mRNA. This takes place with the help of enzymatic reverse transcriptase from a retrovirus. Nucleotides of the mRNA strands are translated to the complementary bases in the following order: A is translated to T, G is translated to C, C is translated to G, and U is translated to A.

Cosmid: A genetic construction to clone large DNA fragments in bacteriophage λ (lambda).

Cross hybridisation: Binding of a DNA fragment to a non-complementary DNA segment. This can take place because several DNA fragments show sequential similarities (homology) and can therefore bind the radioactive fragment if hybridisation conditions do not discriminate sufficiently, i.e. have too low stringency.

Cytoplasm: The viscose content of a eucaryotic cell which is present within the cell membrane, but outside the nucleus.

Cytosol: The part of the cytoplasm that is not inside an organelle.

Diploid: A cell or organism with two complete sets of chromosomes.

D loop (displacement loop): Structure which arises during an early stage of DNA replication.

DNA (deoxyribonucleic acid): The portion of the chromosomes that carry our hereditary characteristics. DNA consists of the four nucleotide bases, adenine, guanine, cytosine and thymine, together with phosphoric acid and the sugar deoxyribose.

DNase: Enzyme which breaks down DNA.

ds (double stranded): Used about DNA or RNA.

Ecosystem: A community of organisms (micro-organisms, plants, animals, etc.) and the abiotic (lifeless) factors in the environment in which the community lives.

Electroporation: A method whereby foreign DNA is introduced into cells with the help of an electric field.

Endocytosis: Uptake of extracellular material by penetration of the cell membrane, which is thereafter drawn together and becomes a vesicle (endosome).

Endonuclease: Enzyme which hydrolyses DNA or RNA by splicing internally in molecules; cf. exonuclease.

Endosome: Membrane-bound vesicle formed by endocytosis.

Endosymbiosis: Dwelling together within another organism to their mutual benefit.

Enhancer: A type of regulatory (control) sequence in eucaryotic DNA which may be located far from the transcription site, above or below, in the gene which it regulates. Linking of specific proteins (transcription factors) to an enhancer will increase or reduce the transcription velocity.

Eucaryote: A cell or organism that has a distinct nucleus; in practice, this means that there is nuclear membrane that delimits the genome from the rest of the cell.

Exon: Part of an RNA molecule that is preserved in the completed, functional mRNA.

Exonuclease: Enzyme which breaks down (hydrolyses) DNA or RNA from one of the endenes (either 5` or 3`).

Expression vector: Plasmid or another type of DNA which is tailored to give high expression of a protein or RNA of interest.

Gene: Hereditary characteristics, i.e. part of a chromosome whose nucleotide sequence codes for a specific protein product.

Genome: The complete set of chromosomes in an organism.

Genotype: A collective term for the entire genetic constitution (genes) of an individual; cf. phenotype.

Genus: A taxonomic group of species into which a family is divided.

G proteins: Guanine-binding (nucleotide) proteins that are linked together by special receptors in the cell membrane. When the receptor links, for example a hormone or a growth factor, G proteins can either send or block an intracellular signal.

Haploid: A cell or individual with only one set of all the chromosomes. Sperm cells and unfertilised eggs are haploid.

Homology: Sequence similarity between two different genes.

Hsp (heat shock protein): The genetic products of genes which gain enhanced expres-sion when the cell is exposed to a rise in temperature or some other stressing treatment.

Hybridisation: Process whereby two single-stranded nucleic acids, RNA or DNA, base pair with one another thereby forming a double-stranded molecule.

Hydrophobic: Used of molecules which do not interact effectively with water, i.e. are insoluble in it. Such molecules can diffuse through cell membranes.

Intron: The part of an RNA molecule that is removed during maturation of an mRNA (cf. exon).

***In situ*:** "In the original position", is used about methods which determine in what types of cells in an organ and where in the cell a nucleic acid or protein is found.

***In vitro*:** "In glass", i.e. in a test tube, or outside an organism.

***In vivo*:** In an organism.

Kbp (kilo base pair): One thousand base pairs; is used as a quantitative term for double-stranded nucleic acids. The term bp, i.e. one thousand bases, is used for single-stranded molecules.

Kinetochore: The area which attaches the chromosome to the spindle fibres**, and** forms part of the centromere. Plays an active role in the movement of the chromosomes towards the poles during cell division.

LCR (ligase chain reaction): Amplification of DNA fragments based on the effect of a thermosta-ble ligase.

Locus: Position of a particular gene on a chromosome.

Lysosome: A small membrane-clad organelle which has low internal pH and contains enzymes that can digest material taken up by phagocytosis and endocytosis.

Microcosm: Small, well-controlled laboratory system which imitates the processes and interactions in a larger, natural ecosystem.

Mismatch: Non-matching nucleotide pairs within a segment of perfectly paired nucleotides.

Mutation: Change in the genes of a cell.

Mycoplasms: Procaryotes which differ from "genuine" bacteria by being smaller and lacking a rigid cell wall. The smallest and simplest organisms that are capable of autonomous growth.

Nano: 10^{-9}, for example gram or mol.

Nematode: Roundworm that may be a parasite in plants and animals.

Nucleoside: A small molecule consisting of an organic nitrogen-bearing base (adenine, thymine, guanine and cytidine) linked to a sugar molecule (ribose in RNA, deoxyribose in DNA).

Nucleotide (nt): Building block of RNA and DNA. Consists of a nucleoside that has one or more phosphate groups linked to the sugar.

Oligonucleotide: A single-stranded DNA molecule with ca. 10-100 nucleotides.

Oncogenes: A group of cellular genes that can lead to the development of cancer.

ORF (open reading frame): The part of mRNA molecules that specifies a genetic product, a protein.

Organelle: Any membrane-bound structure present in the cytosol of a eucaryotic cell.

Origin: Position (sequence) on DNA where replication is initiated.

PCR (polymerase chain reaction): Enzymatic technique for increasing the numbers of a specific DNA or RNA fragment. Between 10^5 and 10^7 identical copies can be obtained from a rarely occurring sequence in the sample.

Phagocytosis: Process that is able to bring relatively large particles into eucaryotic cells. Phagocytosis of infections using special types of cell is part of the immune defence of an organism.

Phenotype: The visible or provable manifestations of an individual's genetic composition.

Phylogeny: Evolution of a species, used in contrast to ontogeny, the development of an individual.

Pico: 10^{12}, for example gram or mol.

Plasmid: Extrachromosomal (episomal), self-replicating DNA molecule.

Point mutation: Change of only a single base pair in a gene.

Procaryote: Cell or organism that lacks a nuclear membrane.

Promotor: A DNA sequence that links RNA-synthesising enzymes (RNA polymerase) and thereby initiates the transcription of a gene.

Protozoans: Non-photosynthesising, single-celled organisms with a clearly differentiated nucleus. Generally considered to be the lowest forms of animal life, although some of them seem to be closer to plants.

Pseudogene: Inactive gene that shows substantial sequential homology with a closely related active gene.

Recombinant DNA: Any DNA molecule formed by linking fragments from different sources.

Recombination: Used about natural processes whereby DNA sequences from different molecules are brought together on the same molecule or chromosome.

Replication: Multiplication of DNA by a process which leads to daughter molecules being identical with the original molecule.

Replication origin: A sequence on a DNA molecule where the replication process begins.

Restriction enzyme: Bacterial enzyme which cleaves double-stranded DNA based on a specific recognition sequence.

Reverse transcriptase: An enzyme in retroviruses which synthesises DNA anew with single-stranded RNA as the template.

Ribosome: A complex that comprises several different RNA molecules and more than 50 proteins and which is organised in one large and one small sub-unit and is the site for protein synthesis.

Rickettsia: Micro-organisms that are smaller than "genuine" bacteria, but which, in contrast to viruses, are visible with an ordinary microscope. Most are parasites which grow intracellularly in a receptive host animal.

RNA (ribonucleic acid): Consists of the sugar ribose, the nucleotide bases adenine, guanine, cytosine, uracil, and phosphate. A cell contains three types of RNA, all of which are involved in protein synthesis: mRNA (messenger RNA), transfer RNA (tRNA) and ribosomal RNA (rRNA).

RNase (ribonuclease): An enzyme which cuts an RNA strand or completely breaks down an RNA molecule to ribonucleotides.

R-plasmid: Plasmid that contains antibiotic-resistant genes and is capable of being spread between bacteria by conjugation.

Segregation: In the context of cell division, this is a controlled process whereby the chromosomes are correctly apportioned among the daughter cells.

Shuttle vector: A plasmid that has the ability to duplicate itself in both procaryotic and eucaryotic cells.

Spiroplasm: Small micro-organisms related to mycoplasm, able to cause diseases in plants and insects.

Splicing of RNA: A process resulting in the removal of introns and the joining together of exons.

Synergism: The effect of working together is greater than the sum of the individual effects of each substance or process.

Taxonomy: Systematic description and arrangement of individuals of species, genera and families or groups.

Telomere: Area on the end of a eucaryotic chromosome containing a characteristic DNA sequence that is replicated by telomerisation. This counters the tendency for a chromosome to be shortened each time it replicates.

TNF (tumour necrosis factor): Protein produced by a series of cells in the immune system and causing the destruction (necrosis) of some types of growth, but principally of severe cases of inflammation.

Transcription: The process by which an RNA copy is produced from a DNA template.

Transduction: Transfer of bacterial genes from one bacterium cell to another with the help of bacteriophages.

Transfection: Introduction of DNA or RNA into a host cell. Usual transfection methods include calcium phosphate co-precipitation, electroporation and lipofection.

Transformation: Similar to transfection, but is used specifically about introduction of nucleic acids to bacteria.

Transgene: A cloned gene which has been introduced and stably incorporated in a plant or an animal and which is passed on to successive generations.

Translation: Translation of the base sequence in an mRNA to the amino acid sequence in a protein.

Translocation: Chromosome disturbance which entails the movement of a segment to a new location on either the same or another chromosome.

Transposon: A DNA element which can exchange position and integrate in bacterial genomes or plasmids irrespective of the recombinant systems of the cell. Some transposons carry genes that can code, for example, for bacterial toxins or resistance to environmental toxins.

Vector: Plasmid, cosmid, bacteriophage, animal virus, YAC, etc., used to transfer foreign DNA to a host organism.

Vesicle: An intracellular "blister" consisting of a membrane-enclosed, fluid-filled cavity.

Viroid: Least known infectious agent of disease. Consists solely of single-stranded RNA. Owing to an extreme number of secondary and tertiary structures, viroids are highly resistant to physico-chemical effects and ribonucleases. Still only known from plants.

Virus: Tiny parasite consisting of a genome, *either* RNA *or* DNA, enclosed in a protein shell. Viruses can only duplicate in a living host cell.

Xenobiotics: Compounds which man releases into the environment in concentrations that cause undesirable effects.

Xenogenesis: Used about transfer astride species boundaries, such as transplants.

***Xenopus laevis*:** Species of frog much used in evolution studies.

YAC (yeast artificial chromosome): Like natural chromosomes, this has two arms. Large DNA fragments can be glued into both arms. YACs can be transformed into both yeast and mammalian cells.