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INTER-AMERICAN STUDY GROUP OF THE NEW
BIOTECHNOLOGY IN AGRICULTURE AND HEALTH

THE IMPORTANCE OF NEW BIOTECHNOLOGY FOR LATIN AMERICA AND THE CARIBBEAN

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1. Introduction and General Remarks

In recent years, we have witnessed a series of scientific revolutions, starting with the computer revolution of the fifties, made possible by the discovery of the transistor, followed by the LASER and integrated circuits revolutions of the sixties and seventies respectively. The decade of the eighties heralded the systematic development of genetic engineering, and it would appear that neither Watson nor Crick, who first reported on the tridimensional structure of deoxyribonucleic acid (DNA) for the first time thirty years back, had any idea of the immense door they had opened for the scientific world. As someone already stated in most graphic terms, an earthquake took place of unprecedented intensity, whose aftershocks were unforeseeable and whose epicenter was the University of Cambridge; an earthquake which aroused the most mixed of human emotions, both at a purely philosophical or religious and a scientific and practical level.

For all intents and purposes, the biotechnological revolution, in all its new cellular and molecular dimensions, is the most recent of latter-day scientific and technological revolutions, despite the fact

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that the Egyptians and the Babylonians, as we all know, were master brewers thousands of years before the current era. What is certain is that the new expressions of this discipline, which draw on the basic modern knowledge of the chemical and biological sciences and of engineering, fueled an industrial boom which already involves more than 200 firms in the United States and 150 firms in Japan, to mention only two of the countries at the forefront of this process. It is estimated that over the course of the next ten to fifteen years these two countries will be investing US$100 billion and US$80 billion respectively in the biotechnology industry. Conservative figures suggest that world sales in biotechnology, which totaled US$25 million in 1983, will exceed US$27 billion in 1990, marking a more than thousandfold increase in under seven years. Moreover, the new products on the market, resulting from genetic engineering research and development, are expected to generate approximately US$400 billion in sales by the year 2000. While there are no less than a dozen definitions of biotechnology or "life technology", as some call it, it is clear that this discipline, whose pillar is genetic engineering, is responsible for new creations and models with untold applications in the near future.

Biotechnology is just one more example of the vertical integration process taking place in today's world, where efforts are under way to acquire new knowledge, fresh out of the laboratory, and convert it into a manufactured product with high market potential. In other words, biotechnology is a perfect example of technological innovation, where even the most basic research and development overlap

in a series of sequential steps which ultimately lead to the same end. For the most part this process has fallen into the hands of powerful private companies which have created high profitmaking expectations in terms of their stock market shares, and successfully so, at least until very recently. Nevertheless, according to a recent article by John Elkington, published by the World Resources Institute, it has been a rude awakening for many of these companies who have learnt that they will have to sit back and wait patiently before enjoying any substantial economic return, despite the size of their investments in many instances. This accounts for the new direction some of these companies are taking, focusing their activities on products which can ensure the most immediate return on their investment. As Elkington points out, this only confirms how little the United States biotechnology industry regards the developing countries as a potential market for its products.

It is worth noting that in 1984 "high tech" accounted for 40 percent of the sales of a company such as DuPont, prompting it to pour US$85 million into a biotechnology research laboratory in Delaware that same year. At that time, DuPont was spending US$200 million of its research and development (R + D) budget on basic research; an additional US$250 will be spent on projects related to the "life sciences". In 1984, Monsanto opened a US$160 million biotechnology laboratory in Missouri, which will create jobs for more than 1000 persons. That same year roughly US$200 million were allocated for biotechnology research. Another noteworthy point is the takeover of G.D. Searle by Monsanto, which intends to open up an even larger

market for the biotechnological products manufactured by the two firms.

2. Prospects for Latin America and the Caribbean

In light of this problem, and by way of contrast, it is important to note that despite the prospects which biotechnology holds for Latin America and the Caribbean, the region is replete with a series of problems. The most outstanding include enormous foreign debt on the order of US$600 billion and soaring inflation, both of which appear to be unmanageable. At the same time, the developed countries show signs of slow economic growth and, despite efforts to slow down the arms build-up, they are nonetheless spending close to US$800 billion annually on arms and defense. All of the foregoing paints a rather bleak picture of what the future has in store for our countries, and seriously casts doubt on their aspirations to achieve real development, based on their integration in the world economy. What is more, everything seems to point to the fact that these new technologies, far from serving to automatically save us from disaster, are more likely to be the source of new mechanisms for creating even greater dependence. But, in spite of this grim forecast, a ray of hope and a solution remain: the unification and solidarity of the countries in the region, which should aspire to development and superior well-being for their people, based on steadfast cooperation which leads to the mastery and appropriation of some aspects of these advanced technologies. What is also required is understanding on the part of the industrialized countries (who have nothing to gain from growing impoverishment of a considerable part of the world

population), who should be open to a new style of cooperation. While this smacks of political discourse, --and I cannot hide that in fact it is a "political" speech in the most favorable sense of the word--, it is perfectly justifiable coming, as it does, from a scientist from the developing world who, for the time being, has left the laboratory to don the hat of practising politician. Furthermore, its raison d'etre becomes clear when one looks at the problem from a "macro" rather than a "micro" perspective, as some insist on doing. It is just that I am convinced that, in the final analysis, the decisions which are taken by our countries in this regard ought to be of a political nature.

2.1 The Advantages of Biotechnology and Necessary Action

Let us take a look now at some of the advantages --and even some of the disadvantages-- which biotechnology could have for Latin America, given that it is properly managed. I might add that to renounce this discipline or to reduce it, in our countries, to no more than a "snobbish" intellectual exercise, would be to occasion a very high social cost, which future generations would have to bear.

In spite of the above, it seems clear that biotechnology, with its wealth of applications, provides a host of opportunities to the poor countries which should not be disregarded. It is also apparent that it is an arduous task to have the countries of the region agree on the key aspects of biotechnology which ought to be pursued, given that each one has its own priorities. Nevertheless, there is common ground on which the countries of Latin America and the Caribbean could

meet and seek joint solutions, either by pooling resources or by way of extensive technological transfer among them. Improved health and environmental conditions are, without a doubt, priorities for all of us. So is the search for greater quantities of better quality foodstuffs and the recycling of all those products, particularly of agricultural origin, which are presently being wasted or lost. Likewise, new and more efficient methods of producing renewable energy should interest many of us.

My point is that it is imperative for the countries of Latin America and the Caribbean to open their doors to biotechnology which could be used to alleviate their problems. And I am referring to biotechnology generated both at home and abroad. First of all it is vital that we league together, with our community of interests, and make the wealthy countries aware of our desire to have access to biotechnological know-how, under equitable terms, discouraging rather than encouraging new forms of dependence, while we strive to consolidate local capacity for R and D and technological management. It is also necessary to identify the social and economic implications of these new technologies and design strategies to mitigate any and every negative effect, searching for cooperation mechanisms, in all these aspects, which are fairer and more balanced. There is no doubt that each country needs to develop local capacity so as to make use of the benefits to be reaped from biotechnology, and that the sum of resources from among the Latin American and Caribbean nations is substantial and vital to the vast number of joint actions which are required. This can be achieved through biotechnology networks between

research centers like those already linking nine countries, promoted by UNESCO and UNIDO under the auspices of UNDP, through specific bilateral or multilateral projects, through the exchange of researchers and postgraduate students, and with the resolute support of international organizations and groups interested in our progress.

A recent study prepared by Cristian Orrego for the OAS notes that at least sixteen different organizations are collaborating in the region, developing different facets of biotechnology and training human resources. I feel it is necessary to effectively coordinate and channel these actions, so that rather than duplicate efforts they come to represent a valuable complement to national efforts under way.

Joint action is also required in terms of the legal aspects concerning property rights and regulations governing the safety and manipulation of the environment, for everything concerning chimeras. While no one to date can confirm or deny the negative effects of genetically manipulated organisms, what is certain is that in countries such as the United States serious precautions are taken to prevent the undesirable effects of releasing these substances into the atmosphere. As a basic measure, our countries ought to adhere to the rules and standards of those countries where these new "creatures" are produced, so that we are not subjected, in the future, to a system of double standards, as was the case in the past with some pesticides and pharmaceutical products.

It is also important to devise market and income distribution mechanisms to stimulate production and consumption of a number of products beneficial to society in this region of 325 million inhabitants. It is vital to create the necessary stimuli, tax incentives and suitable tariff mechanisms which provide for special treatment of those goods produced in the region with a high value added and a sizeable component of local know-how. In this regard, the purchasing power of the State should be used to support national and regional production of biotechnological products according to suitable regulations which promote regional cooperation, based on a clear and distinct policy in this regard.

2.2 Some Unfavourable Factors

One drawback in this scenario is the fact that biotechnology in the industrialized countries, as stated earlier, is increasingly falling into the hands of private groups who are denying access to their know-how and who operate on the basis of the old adage "he who pays the piper calls the tune". Elkington cites the case of Genentech which is currently processing 2,500 biotechnological patents (remember that as of 1981 only 1,372 patents had been registered worldwide in the field of biotechnology). The doors which are being closed with the patenting of genetic properties of plants and seeds which guarantee a specific phenotype, in the United States (Resolution 35 U.S./C.-101 of 1985), is cause for concern, particularly if we consider that the germplasm could conceivably come from our region. This is all the more alarming if we consider that this phenomenon could mark the creation of a caste of "technology landlords", to use

an expression coined by Jorge Yanovsky, which would result in the monopolization, rather than the democratization of this know-how. Equally disconcerting in terms of the generation of new technology is the turn which some basic research is taking --conducted by groups whose only concern is profit or personal gain--. The panorama becomes even more complex if we consider that some of our primary exports could be replaced on the market by new substitutes produced in biotechnology laboratories. This is in fact the case with the new sweeteners (aspertame and fructose). The latter is a genetically manipulated microorganism which contains the three enzymes required to convert corn starch to high fructose corn syrup in a single reactor. At least two companies, one American, the other Japanese, have patented both microbial mechanisms for cocoa butter production which, thus far, has not proven to be a profitable endeavour in economic terms.

2.3 Promising Areas

Let us turn now to the positive aspects of biotechnology where I believe our countries can benefit substantially, or at least reap some advantages.

2.3.1 Health

In this area we need to prepare vaccines against tropical diseases which affect both man and domestic animals, using available genetic engineering mechanisms. These should not only include methods whereby a synthetic gene can be introduced in a bacterial plasmid to achieve a desired result, but also processes to synthesize the

determining immunogenic determinant which, in the case of some viruses, as we all know, can turn out to be short-chained peptides. For example, rather than citing the parasite-induced tropical diseases, let us recall that the diseases which are primarily responsible for the high human morbidity and mortality rate in Latin America are acute and chronic respiratory infections produced by different types of viruses. These diseases, which are far less significant in the industrialized countries, not only result in a high number of casualties but occasion extremely high health care costs. For that reason, the production of an effective vaccine, among several of our countries, would prove most desirable.

A greater number of improved methods should also be devised for diagnosing different human and animal diseases, making use of antigens and monoclonal antibodies which allow for greater sensitivity and specificity in reactions (diagnostic kits). Similar efforts are already under way in Brazil and Argentina, and an attempt should be made to arrive at some sort of agreement to ensure that work is divided, not duplicated, with actions complementing one another based on the capabilities of each countries. The same could be said regarding the production of such substances as insulin, interferon and interleukin, where serious efforts are also in progress in the region. We should furthermore consider the production of certain drugs and other substances through biotechnological processes, including the purification and synthesis of certain molecules which are of extreme interest to human and animal health, or of general use to industry, such as amino acids, nucleotides, steroids, enzymes and hormones. All

of the foregoing actions should bear in mind market and marketing conditions in our region and the economies of scale which could be involved.

2.3.2 Agriculture

In the short term, given the need to ensure food security for present and future generations, it would appear that agriculture and livestock production is where biotechnology can make its greatest contribution in Latin America and the Caribbean. Despite prevailing limitations, due in particular to a lack of sufficient basic knowledge in some fields, available empirical techniques are useful, if only for reducing the costs of producing a number of agricultural products which are becoming increasingly indispensable both as a source of food and strong foreign currency. This new possibility of obtaining less expensive and higher quality foodstuffs by biotechnological means contrasts sharply with the technologies of the Green Revolution which are burdensome and oftentimes ecologically unsound.

Some regional agricultural organizations have already taken serious steps towards introducing biotechnology techniques and have enjoyed some success, worthy of a well-defined policy in each country to take maximum advantage thereof and ensure effective dissemination. In terms of tropical agriculture, two areas which should be pursued in the coming years are: a) genetic improvement in plants, either by means of genetic engineering or more conventional methods such as somaclonal micropropagation, haploid anther cultures, cellular hybridization, to increase their resistance to stress-producing

diseases or unfavourable environmental conditions or simply to rid them of harmful viruses, as occurs with meristem cultures, or to increase their photosynthetic, productive or nutritive capacity; and b) manipulation of different microorganisms to improve nitrogen fixation or absorption of phosphorus, to produce biological insecticides to control certain diseases and stimulate growth.

It appears easy to conclude, particularly in light of the advances taking place every day in laboratories across the world, that within five to ten years agriculture will be governed entirely by biotechnology and that our countries cannot be excluded from this process. What is more, because our agriculture is tropical and our diet highly specific, much of the basic and applied research and technological development should take place in our very own region. One example of these efforts, based on the nutritional needs of the region, is the experiment being conducted in the International Potato Center in Peru by staff researchers in collaboration with others scientists from the State University of Louisiana. Using genetic engineering techniques they have succeded in synthesizing a gene which induces production of a protein rich in essential amino acids, which they in turn have succeeded in inserting in the genome of the potato by way of Agrobacterium rhizogenes, as the manipulated plasmid vector. The ultimate objective of the experiement is to directly obtain high protein content in the tuber with the concomitant social gains of such an achievement.

In the domain of livestock production, local efforts should be undertaken, under our own conditions, both in the field of diagnosis, prevention and control of animal diseases, as stated above, and in the area of nutrition, promotion of growth and genetic improvement, by means of techniques which include embryo transplants or multiplication and gene transfer.

Another important step is the use of such techniques as cell cultures and cloning to repopulate our forests which have been terribly degraded in some regions. This would allow us to reduce the time required for plant life to grow and mature, and to appropriately select and reproduce specimens of great genetic value.

We should recall that all of these techniques provide valuable opportunities for the conservation and protection of plant wildlife and domestic flora, as well as endangered animal wildlife.

The conservation of plant germplasm by means of biotechnological processes and cryopreservation, with all the natural benefits derived in terms of physical space, is worthy of special attention, especially in view of what has been referred to as the "seed war" or genetic imperialism. The problem of germplasm from our countries, which is part of our natural resource heritage, should be approached from a different and more global perspective, in an effort to rationalize its use and ensure greater equity to benefit us all. We must also exercise extreme caution and preserve those wildlife species which are required to improve other exsiting species or new varieties. This

should be done for the purpose of preventing "genetic erosion" for the sake of "new plants" which could easily fall prey to some pests.

2.3.3 Other Areas

Several agroindustries could benefit from biotechnology techniques which allow them to upgrade the efficiency and quality of many of their products. Furthermore, advantage could be taken of a number of materials of agricultural origin which presently constitute waste products -- often the cause of serious environmental contamination. At this point reference should also be made to the production of valuable sources of energy such as different alcohols, methane and liquid hydrocarbons.

One important issue is the biodegradation of toxic or undesirabale products with the use of genetically manipulated microorganisms. Some experiments in the biodegradation of oil spills, herbicides and toxic residues are extremely promising. Nevertheless, we are fully aware, as stated earlier, of the possible consequences of releasing these organisms into the atmosphere. However, along similar lines, the use of highly specific microbes to combat harmful insects or some plant diseases, substituting chemical pesticides, is extremely interesting for our countries, given their diversity and ecological complexity.

Equally worthy of notice for Latin America and the Caribbean are biotechnological applications in such disciplines as metallurgy, particularly with regard to leaching of some metals which are

extremely important to several countries in the region. The same could be said of the possible applications of biotechnology for the region's abundant aquatic life, where significant progress has already been made in research centers located in some countries.

3. Final Remarks

As for the future of biotechnology in Latin America and the Caribbean, it remains absolutely clear that our countries must strive, within the framework of a clear and well-defined policy, towards using existing techniques in select areas of extreme mutual interest. At the same time they should further develop their local capacity to master these techniques and develop their own, based on extensive multidisciplinary research which includes basic apsects, particularly in the field of tropical agriculture and allied basic disciplines. Most of this research should be conducted or strengthened in multinational institutes like those already located in the region, and should complement, not replace, the excellent agricultural research already being conducted. We should furthermore urge our Latin Aemrican universities to strengthen their research teams in the basic sciences which nurture biotechnology, to train duly prepared personnel and to play a more active role, together with the government and the productive sector, in development programs aimed at the production of biotechnological products. Concerted government and university action, to create and strengthen molecular biology and genetic engineering institutes, and a similar brand of action among governments, universities and the private sector, to join forces in the creation of new ventures in science parks under the coordination

of the State, seem most appropriate for the region. The latter would be responsible, amongst other things, for elaborating quality biotechnolgical products with a high degree of value added, which take into account the socioeconomic needs of the region.

Part of the scientific-technological transfer process in these cases could be carried out by means of joint ventures, with groups from the region or outside, within a framework of fairness and assimilation, reflecting the interests and priorities of the region. The governments should also provide the necessary incentives, including venture capital, for investors interested in participating in this type of undertaking or new opportunity. At all times a balance must be struck between the search for profit and the needs of our societies.

The recent "Compromiso de Acapulco para la Paz, el Desarrollo y la Democracia" signed by ten Latin American Heads of State whose countries constitute the member countries of the "Mecanismo Permanente de Consulta y Concertación Política", in turn an outgrowth of the Contadora and Support Groups, states that: "Regional integration is a political commitment of capital importance to our countries and an instrument for change and modernization which ought to engage the active participation of all economic and social factors." It goes on to refer to a series of economic measures in the region the basic objective of which, in the final analysis, is to move towards a Latin American common market. Lastly, they devote two clauses to science and technology, establishing the need to: "promote a program of

association and cooperation in science and technology, to achieve technological autonomy in priority areas, particularly in the domain of advanced technology".

Thus, it is only by joining forces that we can make ample and appropriate use of biotechnology, as an expression of advanced technology, to meet the needs of our people and to build bridges between our countries to benefit the most needy. The common language of biotechnology will allow us, as a community, to tackle common problems and improve the quality of life for all. That is why it is fitting to ask ourselves whether biotechnology is not the new Simon Bolivar who will make us recognize the need for strong material and intellectual unity, and help us eradicate some of the social ills which unfortunately still beset us? Or better still: Will we, due to our inability to achieve solidarity and integration, jeopardize this opportunity to benefit our people, allowing history to be our judge? Only time will tell.

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The Use and Safety of Genetic Engineered Techniques

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NEW vs. CONVENTIONAL "BIOTECHNOLOGY": THE CORRECT PERSPECTIVE

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"OLD" BIOTECHNOLOGY AND "NEW" BIOTECHNOLOGY: A PERSPECTIVE

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INTRODUCTION

The purpose of this Inter-American Study Group project, "New Biotechnology in Agriculture and Health: The Use and Safety of Genetic Engineering Techniques," is, in part, to consider "risk analysis" or "risk assessment" for environmental release of live organisms. The purpose of such risk assessment is to provide scientists, government regulators and others a measure of the safety attendant to the testing or use of a product, and to provide guidance as to the management of the risk that may be present.

According to Fiksel and Covello[1], the risk assessment literature generally defines "risk" as the potential for adverse consequences of an event or activity. Risk assessment or analysis is the process of obtaining quantitative or qualitative measures of risk levels, including estimates of possible health and other consequences. Implied, of course, are approximations of the uncertainty of those estimates.

The components or the techniques of risk assessment may be found elsewhere[2]. We will attempt to place new and old biotechnology in perspective, emphasizing that:

- Biotechnology is not a discrete, unitary entity.
- New biotechnology is not as radically novel as is often portrayed.
- Vast experience with macroorganisms and microorganisms manipulated by nature or by man provide us an important and useful perspective for current and future application, both scientific and commercial.
- The safety provided by governmental regulation can be bought only at a cost, and there is a point at which more stringent regulation and more expenditure of resources do not confer enhanced safety.
- There exist classes of proposed trials -- and certainly individual experiments -- that do not require risk assessments by governmental authorities on each and every proposal.

The goal of FDA's approach -- and, indeed that of the entire U.S. Government's coordinated framework for the regulation of biotechnology products -- is to limit potential risks, while encouraging the innovation, development and availability of new biotechnology products. We must recognize, however, that not only must products be safe, but the public must have confidence in their safety. In this respect,

the advent of new biotechnology poses a major challenge, a challenge that involves perception as much as reality. Only if we can correct misconceptions and eliminate the harmful myths that have grown up around the buzzwork "biotechnology" can the potential of the new technology be realized.

THE MYTHS

Myth No. 1: That Biotechnology is a Discrete Entity

One myth is that biotechnology is something discrete or homogeneous, a corollary of which is that there exists a "biotechnology industry" that can or should be rigidly controlled. This view is facile but inaccurate. Biotechnology is merely a catch-all term for a broad group of useful, enabling technologies with wide and diverse applications in industry and commerce. A useful working definition used by several U.S. government agencies is, "the application of biological systems and organisms to technical and industrial processes." This definition encompasses processes as different as fish farming; forestry; the production of enzymes for laundry detergents; and the genetic engineering of bacteria to clean up oil spills, to kill insect larvae, or to produce insulin. Biotechnology is myriad dissimilar processes producing even greater numbers of dissimilar products for vastly dissimilar applications. Biotechnology processes and products are arguably so diverse and have so little in common with one another that it is difficult to construct valid generalizations about them, for whatever purpose. Putting this another way, biotechnology has no systematic, uniform characteristics that enable it to be legislated or overseen in the homogeneous way that is possible, for example, for the underground coal mining industry.

The diversity of biotechnology has important implications. It dictates that regulation of so many end uses must be accomplished by many government agencies. As the ultimate characteristics and uses of biotechnology's products vary, so does agency jurisdiction over those products (see, for example, references 3-4). And so, of course, does the nature and depth of agencies' evaluation of the products: clearly, EPA's review of an enzyme used as a drain cleaner will be different from FDA's review of the same enzyme injected into patients to dissolve blood clots. The diversity of products and their applications argues against the usefulness of legislation or regulations that attempt to encompass unnatural groupings such as "biotechnology," "genetic engineering," or "deliberate releases"[5].

Myth No. 2: That Biotechnology and Genetic Engineering are New

A second myth is that biotechnology is new. On the contrary, many forms of biotechnology have been widely used for millennia. Earlier than 6000 B.C., the Sumerians and the Babylonians exploited the ability of yeast to make alcohol and brewed beer. A "picture" of the ancients preparing and fermenting grain and storing the brew has even been retained for posterity in a hieroglyphic[6].

Perhaps the best publicized subset of biotechnology -- and also one of the oldest -- is "genetic engineering," the manipulation directly or indirectly of an organism's DNA. Genetic engineering dates from man's recognition that animals and crop plants can be selected to enhance desired characteristics. In the traditional, or "conventional," breeding and selection of improved plants, for example, the genetic material of plants is combined to create new and useful traits. In this well-established form of genetic engineering, changes are made at the level of the whole organism; selection is made for desired phenotypes, and the genetic changes, most often poorly characterized, occur concomitantly. During the past decade or so, new technologies have been developed that enable genetic material to be modified at the cellular and molecular levels, and are more precise and deliberate variants of genetic engineering; the precision of these techniques often provides a better characterized and more predictable product.

Hardy and Glass[7] have carefully distinguished three modes of genetic engineering that constitute a continuum of scientific sophistication and precision: whole organism, cellular, and molecular genetic engineering. They point out that in all three, DNA is modified or combined to increase genetic variation, thereby enlarging the pool of potentially useful traits. The three modes differ not in the end product but in the process used to generate the genetic variability.

In whole organism engineering, or traditional breeding, the genetics of the process is largely random -- entire sets of genes of two animals or plants are combined, with selection for a desired phenotype; concomitant genetic changes are complex and most often poorly-characterized. Despite the relative slowness and laboriousness of this technique, the successes have been monumental, for both interspecific hybridization and intergeneric gene transfer applications. The exploitation of interspecific hybridization for crop improvements is epitomized by the advances in wheat breeding[8]. Gene transfer from related species into cultivated wheat began in 1930, when McFadden reported transferred resistances to stem rust and loose smut diseases from tetraploid emmer wheat to hexaploid bread wheat variety[9]. The resulting bread wheat variety was widely grown in

the U.S. and was responsible for one of the longest rust-free periods in the history of the U.S. wheat cultivation. Other genes for resistance to stem rust and powdery mildew and to Hessian fly have since been incorporated into a number of bread wheat varieties. Recent applications of interspecific gene transfer include successful wide hybridization between the cultivated soybean and its wild perennial relatives[10]. Such successful interspecific transfer of traits from wild species to domesticated relatives in the same genus inspired attempts at even more distant crosses, including those between members of different genera. There is certainly evidence that some of our modern crop species, such as rapeseed, tobacco, and wheat, originated in nature by hybridization between different species or genera, and intentional crosses between species in different genera have also successfully transferred specific traits into crop species. Examples include hybridization between cultivated wheat and species of wild grasses from the genera **Aegilops**, **Agropyron**, and **Secale**, in order to transfer traits such as salt tolerance and disease resistance into the crop[11]. There are no known examples of genetically engineered commercial crop species being transformed by these genetic manipulations into troublesome weeds.

The successes of whole organism genetic engineering have not gone unnoticed. The genetic engineering of wheat for human consumption was recognized by the Nobel Peace Prize in 1970 and the engineering of rice by the Japan Prize in 1987 (ref. 12).

A marked improvement over whole organism genetic engineering, the cellular and molecular technologies provide opportunities for unprecedent precision and specificity, because smaller amounts of better characterized genetic information can be transferred than is possible in a traditional animal or plant cross.

Cellular genetic engineering, which uses the techniques of cell culture and cell fusion to create genetic variation, is less random than engineering at the whole organism level. Thus, fewer variants are necessary to produce organisms with the desired properties, and their selection is easier than at the whole organism level. However, cellular genetic engineering also has limitations: specific genetic changes that result from the engineering may not be known, and there may be changes other than those that confer the desired properties. Cell culture is expected to have a major beneficial effect on agriculture; commercially valuable crops such as sugarcane can be regenerated from cell culture, although this has yet to be achieved for other important crops such as wheat, corn, and soybeans[6].

Molecular genetic engineering employs recombinant DNA and related techniques for genetic constructions.

The actual transfer of DNA into plants may be accomplished in several ways: mediated by the Ti plasmid of **Agrobacterium tumefaciens**; direct DNA transfer from culture medium; microinjection into plant protoplasts; or mediated by virus-based gene expression systems. The molecular techniques make possible a highly specific and precise approach to genetic engineering; the genetic material transferred from one organism to another is usually completely characterized, often consisting of a single gene coding for a hormone or other protein. Molecular genetic engineering has several advantages over whole organism or cellular genetic engineering: specificity and precision of the genetic change; fewer variants created in order to obtain an organism with desired characteristics; the ability to perform experiments in a short time; and the ability to insert synthetic genes (that may not exist at all in nature).

During the past half-century, increased understanding of genetics at the molecular level has added to the sophistication of the genetic engineering of microorganisms. An excellent example is the genetic improvement of **Penicillium chrysogenum**, the mold that produces penicillin: by several methods including screening thousand of isolates and mutagenesis, penicillin yields have been increased more than a hundred-fold during the past several decades. There are many similar examples; microbial fermentation is employed throughout the world to produce a variety of substances including industrial detergents, antibiotics, organic solvents, vitamins, amino acids, polysaccharides, steroids, and vaccines[13]. The value of these products is in excess of $5 billion annually.

In addition to such contained industrial applications, there have been "deliberate releases" of other organisms, including insects, bacteria, and viruses. There are innumerable examples of successful and beneficial "releases," or uses, of live organisms in the environment. Insect release was used successfully to control troublesome weeds in Hawaii early in the 20th century[14]. Other examples are the highly successful program for biological control of St. Johnswort ("Klamath weed") in California by insects in the 1940's and 1950's, and the more recent use of an introduced rust pathogen to control rush skeletonweed in Australia. This remains an area of active research that is promoted by a permanent Working Group on Biological Control of Weeds, overseen jointly by the U.S. Departments of Interior and Agriculture[14].

Currently, more than a dozen microbial pesticidal agents are approved and registered with the U.S. Environmental Protection Agency, and these organisms are marketed in 75 different products for use in agriculture, forestry, and insect control[15]. In another major area, bacterial preparations containing **Rhizobium** that enhance the growth of leguminous

plants (e.g., soybeans, alfalfa, beans) have been sold in this country since the late 19th century; these products allow the plants to produce nitrogen fertilizer from the air.

The most ubiquitous "deliberate releases" of genetically engineered organisms have been during vaccination of human and animal populations with live, attenuated viruses. Live viruses engineered by various techniques and licensed in the U.S. include mumps, measles, rubella, poliovirus, and yellow fever. Inoculation of a live viral vaccine involves not only infection of the immediate recipient, but the possibility of further transmission of the virus and its serial propagation in the community. It is notable that none of the vaccine viruses has become established in the environment, despite their presence there. For example, the presence of vaccine strains of poliovirus in sewage in the U.S. and U.K. reflects the continuing administration and excretion of live virus vaccine rather than its serial propagation in the community [16]

Viral vaccines produced with older genetic engineering techniques have been awesomely effective throughout the world; they are rivaled only by the agricultural "green revolution" as a promoter of human longevity and quality of life. The newest biotechnological techniques, including recombinant DNA, are already providing still more precise, better understood, and more predictable methods for manipulating the genetic material of microorganisms for vaccines.

An analogy is sometimes made between the possible consequences of introducing into the environment organisms manipulated with the new genetic engineering techniques and the ecological disruptions that have been caused by the introduction of certain nonnative (alien or "exotic") organisms; examples cited often include the gypsy moth, the starling, and the kudzu vine. However, this comparison is specious, depending largely on the assumption that rDNA manipulations can alter the properties of an organism in a wholly unpredictable way that will cause it to affect the environment adversely. As discussed below, both theory and experience indicate that this is very unlikely. Genetically manipulated organisms, whether engineered by conventional or new molecular techniques, closely resemble the parent organism, and are, in fact, often at an evolutionary disadvantage with respect to their parents and cohorts.

Myth No. 3: "The unknowns far outweigh the knowns where the ecological properties of microbes are concerned." [17]

This is an excessively negative statement, and is particularly dubious for many microorganisms of commercial interest, including **Pseudomonas syringae, Thiobacillus** species,

Bacillus thuringiensis, **Bacillus sutilis**, **Rhizobium**, and **Baculovirus**, to name a few. Also, it should be noted that many microbes are essential to ecosystem processes or otherwise beneficial to man, and that only a minuscule fraction of microbes are pathogenic or otherwise harmful. In the heading above, one could just as easily substitute "the functions of the mutations in polio virus vaccine" for "the ecological properties of microbes;" these unknowns have not prevented our using live, attenuated polio virus vaccine safely and effectively for three decades. Similarly, they have not prevented the unregulated small-scale testing of innumerable different microbes in the environment -- small-scale field trials were exempt from both the U.S. pesticide and toxic substances statues until recently -- and which boasts an admirable safety record. The scientific method and prior experience applied logically to risk assessment do enable us to make useful predictions.

Myth No. 4: That New Genetic Engineering Techniques Will Create Novel, Dangerous Organisms

The degree of novelty of microorganisms or macroorganisms created by the new genetic engineering techniques has been widely exaggerated. A corn plant that has incorporated the gene for and synthesized the **Bacillus thuringiensis** toxin is still, after all, a corn plant. **E. coli K-12** that has been programmed to synthesize human interferon alpha by means of recombinant DNA techniques really differs very little from its unmanipulated siblings that manufacture only bacterial molecules. Moreover, nature has already tried out innumerable recombinations between even very distantly-related organisms, via several mechanisms (see, for example, ref. 17). Bacteria in nature have long been exposed to DNA from lysed mammalian cells -- for example, in the gut, in decomposing corpses, and in infected wounds. The human population alone excretes on the order of 10^{22} bacteria per day; hence, over the past 10^6 years, many mammalian-bacterial hybrids are likely to have appeared and been tested by natural selection. An analogous argument can, of course, be made for recombination among fungi, bacteria, viruses, and plants. Kilbourne[16] has emphasized that both genetic and ecological constraints operate to prevent the emergence of hyper-virulent viral variants, even though single point mutations can alter virulence. And while nature does occasionally produce, on a time scale that we can observe, a modified pathogen (such as an influenza virus with increased virulence, or HIV-1), we must ask how likely it is that it would do so in one fell swoop from a non-pathogen [**vide infra**]; the chances of such an event arising from the small-scale man-made changes must be compared with the tremendous background "noise" in nature.

Myth No. 5: That Genetic Manipulation Will Transform a Non-Pathogen into a Pathogen

An often-mentioned concern is that genetic manipulation may inadvertently transform a non-pathogen into a pathogen. However, this view ignores the complexity and the multi-factorial nature of pathogenicity. Pathogenicity is not a trait produced by some single omnipotent gene; rather, it requires the evolution of a special set of properties that involve a number of genes. A pathogen must possess two general characteristics, which are themselves multi-factorial. First it must be able to metabolize and multiply in or upon host tissues; that is, the oxygen tension and pH must be satisfactory, the temperature suitable, and a favorable nutritional milieu available. Second, assuming an acceptable range for all of the many conditions necessary for metabolism and multiplication, the pathogen must be able to resist host defense mechanisms for a period sufficient to reach the numbers required actually to produce disease. Thus, the organism must be meticulously adapted to its pathogenic life-style, and even a gene specifying a potent toxin will not convert a harmless bacterium into an effective source of epidemics -- or even localized disease -- unless many other required traits are present. These include, at the least, resistance to host defenses, ability to adhere to specific surfaces, and the ability to thrive on available nutrients provided by the host. And although no one of these traits confers pathogenicity, a mutation that affects any essential one can eliminate it. Moreover, **severe** pathogenicity is more demanding, and much more rare in nature than mild degrees of pathogenicity, and so the probability of inadvertently creating an organism capable of a medical catastrophe must be vanishingly small.

Myth No. 6: That all Technology is Intrinsically Dangerous

Another myth is that the application of all new technology is dangerous. The bases of this are probably atavistic fears of disturbing the natural order and of breaking primitive taboos, combined with the complexity for the non-scientist of the statistical aspects of risk. The promulgators of this myth who seek to discredit biotechnology eagerly cite the hazards of toxic chemical waste dumps and the technical problems of the nuclear industry, but conveniently ignore the overwhelming successes of telephonic communication, vaccination, blood transfusions, microchip circuitry, and the domestication of animals, plants, and microbes. It is worthy of note that some predicted electrocution from the first telephones, the creation of human monsters by Jenner's early attempts at smallpox vaccination, and the impossibility of matching blood for transfusions. They said, in effect, "The costs will be too high, there is no such thing as a free lunch."

No responsible person would suggest that some novel practices, processes or products of biotechnology could not be hazardous in some way. Some of these are already well known: workers purifying antibiotics have experienced allergic reactions; beekeepers have been stung; laboratory workers have inadvertently sucked up bacteria through a pipette and suffered gastroenteritis or worse; and vaccines occasionally elicit adverse reactions.

In addition, there have been examples of introductions of exotic species, such as English sparrows and gypsy moths, that have had serious economic consequences. However, the introduction of exotic species is not a useful model for the kinds of organisms being contemplated with new biotechnology. Generally, introductions will be of indigenous organisms that differ minimally (often by only the insertion or deletion of a single structural gene) from organisms already present in the environment, and that will not enjoy a selective advantage over their wild-type cohorts. They will be subject to the same physical and biological limitations of their environments as their unmodified parents. As noted above, a corn plant that has incorporated the gene for and synthesizes the Bacillus thuringiensis toxin is, after all, still a corn plant. Thus, a more applicable model for organisms modified by the techniques of new biotechnology is the selective breeding and testing of domesticated plants, animals and microbes, with which there is vast experience and which boasts an admirable safety record.

In any case, a complex and comprehensive regulatory apparatus based in numerous federal agencies in the U.S. has long overseen the safety of food plants and animals, pharmaceuticals, pesticides and other products that can be produced by biotechnology. This should continue to be equal to the task, and to perform in a way that does not stifle innovation. There may never be a "free lunch," but often we can make it an excellent value.

THE APPLICABILITY OF RISK-ASSESSMENT METHODS FOR ENVIRONMENTAL APPLICATIONS OF BIOTECHNOLOGY

Among those scientifically knowledgable about the new methods of genetic manipulation, there is wide consensus that existing risk assessment methods are suitable and applicable for environmental applications of new biotechnology. Several appropriate risk assessment alternatives are available, including: deterministic consequence analysis with confidence bounds; qualitative screening; and probabilistic risk assessment[2]. While it is true that risk assessment is not an exact, quantitative, and predictive discipline, we agree with a National Science Foundation report's conclusion that available

methods provide a useful foundation and "a systematic means of organizing a variety of relevant knowledge about the behavior of microorganisms in the environment"[2]. The need for risk assessment of new biotechnology is not new methods, but rather in ascertaining the correct underlying assumptions. For risk assessment as for many other aspects of new biotechnology, new products manufactured with new processes do not necessarily require new regulatory or scientific paradigms.

The above view is supported by the recent report of the U.S. National Academy of Sciences, "Introduction of Recombinant DNA-Engineered Organisms into the Environment: Key Issues[19]." This landmark report has wide-ranging implications in the international community, by providing an authoritative perspective on planned introductions. Several of the most significant of its conclusions and recommendations are:

- R-DNA techniques constitute a powerful and safe new means for the modification of organisms;
- Genetically modified organisms will contribute substantially to improved health care, agricultural efficiency, and the amelioration of many pressing environmental problems that have resulted from the extensive reliance on chemicals in both agriculture and industry;
- There is no evidence that unique hazards exist either in the use of rDNA techniques or in the movement of genes between unrelated organisms;
- The risks associated with the introduction of rDNA-engineered organisms are the same in kind as those associated with the introduction of unmodified organisms and organisms modified by other methods; and
- The assessment of risks associated with introducing rDNA organisms into the environment should be based on the nature of the organism; based on the environment into which the organism is to be introduced; and independent of the method of engineering **per se**.

The conclusions and recommendations of the National Academy of Sciences report have been echoed elsewhere. Examples include the report on a NATO (North Atlantic Treaty Organization) Advanced Research Workshop, held in Rome in June 1987 ("Recommendations for a Scientific Approach to Safety Assurance for Environmental Introductions of Genetically-Engineered Organisms," submitted for publication); and the results of a conference held in Bellagio

in September 1987 ("Introduction of Genetically-Modified Organisms Into the Environment: A Statement from the Scientific Committee on Problems of the Environment (SCOPE) and the Committee on Genetic Experimentation (COGENE)").

We can summarize the current situation regarding the regulation of new genetic engineering products in a syllogism. Industry, government, and the public already have considerable experience with "deliberate release" of traditional genetically engineered products, including **Rhizobia** for agriculture and live vaccines such as measles and polio. Existing regulatory schemes have protected human health and the environment while simultaneously stimulating industrial innovation. As noted above, there is no evidence that unique hazards exist either in the use of rDNA techniques or in the movement of genes between unrelated organisms. **Therefore, there is no need for additional regulatory mechanisms to be superimposed on pre-recombinant-DNA regulation.**

IMPLICATIONS FOR GOVERNMENTAL POLICY-MAKERS

Of concern to many practitioners and regulators of biotechnology are two non-scientific issues that could play a dominant role in the future of biotechnology development and use in the U.S. and abroad. First, the regulatory climate could, if not rationalized or if inappropriately risk-averse, impede industry's eventual introduction of products being developed now. This could lead to future withdrawal or diminution of new product development, with continued reliance on less sophisticated -- and often more hazardous -- alternative technologies. Second, concerns within the financial community about the long term stability and success of companies doing business in environmentally regulated fields could depress company values, drying up capital for the continued development and testing necessary to satisfy regulatory requirements[19].

Those who are associated with the regulatory issues of biotechnology have a critical responsibility to act quickly and definitively to balance the various opposing forces facing this technology. We reject the notion that all products derived from new biotechnology defy useful, accurate risk assessments or are too dangerous to introduce into the environment: both theory and experience repudiate this assertion. We must be guided in part by the knowledge that there are genuine costs of overly risk-averse regulatory policies that **prevent** the testing and approval of new products; crops destroyed by frost and the continued application of dangerous chemical pesticides while the "ice-minus" bacteria and new bio-rational pesticides languish untested for years represent a significant toll. At the same time, we must acknowledge legitimate concerns about the safety

of product testing and use. The principles that govern the safe use of these products and allow the underlying research to proceed must evolve and be refined. In such a fast-moving technological environment, it is necessary to reappraise regularly the scientific basis of existing regulation and to make any required adjustments in either the technology of regulation or the statutory basis for regulation.

The professional practitioners, regulators and observers of biotechnology must strive to demystify it and to provide the proper perspective for the public, because it is the public who will benefit most. The stakes are high both in economic terms and in terms of social benefit. In the past year, the U.S. Food and Drug Administration has approved several products of new biotechnology that are medical milestones. These include alpha-interferons for the treatment of a lethal leukemia, a monoclonal antibody preparation for preventing rejection of kidney transplants, and a new-generation hepatitis vaccine. Among myriad other applications, biotechnology promises vaccines against scourges such as malaria, schistosomiasis, and AIDS, and new therapies that could ameliorate or cure for the first time such genetic diseases as sickle-cell anemia or certain inherited immune deficiencies. Used for new generations of medicines and food plants and animals, it could provide partial solutions to the trinity of despair -- hunger, disease, and the progressively deteriorating mismatch between material resources and population.

SUMMARY

Biotechnology and its subset, genetic engineering, have been widely applied for millenia, including innumerable successful and beneficial uses, or "releases," in the environment. The precision and power of genetic manipulation of both macroorganisms and microorganisms have increased during the past half century with increased understanding of molecular genetics. The techniques of "new biotechnology" are generally viewed in the U.S. as extensions -- refinements -- of older techniques for genetic manipulation. For these reasons, among those scientifically knowledgable about the new methods of genetic manipulation, there exists wide consensus that current risk assessment methods are suitable and applicable for environmental applications of new biotechnology; new products manufactured with new processes do not necessarily require new regulatory paradigms. Finally, there are genuine costs of overly risk-averse regulatory policies that prevent the testing and approval of new products; such policies are anti-innovative, anti-competitive, and delay the benefits of the new products to the public. These policies both emanate from and feed a pernicious anti-science movement that threatens basic scientific research as well. The long term remedy must be improved public education about science and technology to produce a generation with at least enough knowledge to avoid being "bamboozled by foolishness."[20]

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The Use and Safety of Genetic Engineered Techniques

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BIOTECHNOLOGY AND VETERINARY MEDICINE

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In less than 35 years, deoxyribonucleic acid (DNA) has become a central feature of research in many different areas of veterinary medicine. So rapidly has the knowledge of DNA and its implications been accumulated that many veterinarians feel isolated from these developments.

This lecture focuses on biotechnology which can be broadly defined as any technique that uses living organisms to make or modify products, to improve plants or animals, or to develop micro-organisms for specific uses.[113]

The history of biotechnology goes back to man's earliest days. Our ancestors didn't realize a few thousand or so years ago, when they let a jug of wine or beer ferment, that they were indeed biotechnologists. It is difficult to pick out the most important milestones, but we should begin with Miescher at the University of Tubingen in West Germany. In 1869 he isolated a new substance from nuclei of fish cells which he called "nuclein". It was later labeled nucleic acid. Mendel's 1865 paper, in which he established the laws of genetics, was ignored until 1900.

The first definitive evidence that DNA was the carrier of genetic information was published by Avery, MacLeod and McCarty in 1944. A DNA fraction isolated from heat killed Type III pneumococci transformed unencapsulated "rough" Type II pneumococci into fully encapsulated Type III cells and the change was permanently heritable.[108]

In 1953, Watson and Crick used less than a thousand words to unveil the double helix of DNA which ushered in the age of molecular biology and revolutionized the study of living things.[114] DNA contains all the information to reproduce any organism from a flea to an elephant. Their work deserves a great deal more than the cursory explanation that follows.

DNA is packaged in the chromosomes of a cell; its structure resembles a long spiral staircase or twisted zipper. The teeth on the zipper consist of 4 subunits of DNA, the nucleotide bases (adenine, thymine, cytosine and guanine) that fit together in a precise fashion (Fig. 1).

DNA replicates by separation of the two parallel strands, often called backbones, that attach to the zipper's teeth. This "unzipping" leaves each strand with its teeth (bases) to serve as a pattern for the assembly of another strand identical to the one from which it separated (Fig. 2). A gene is actually a piece of DNA comprised of hundreds or thousands of nucleotides that direct the assembly of amino acids in the making of a protein.[96,115]

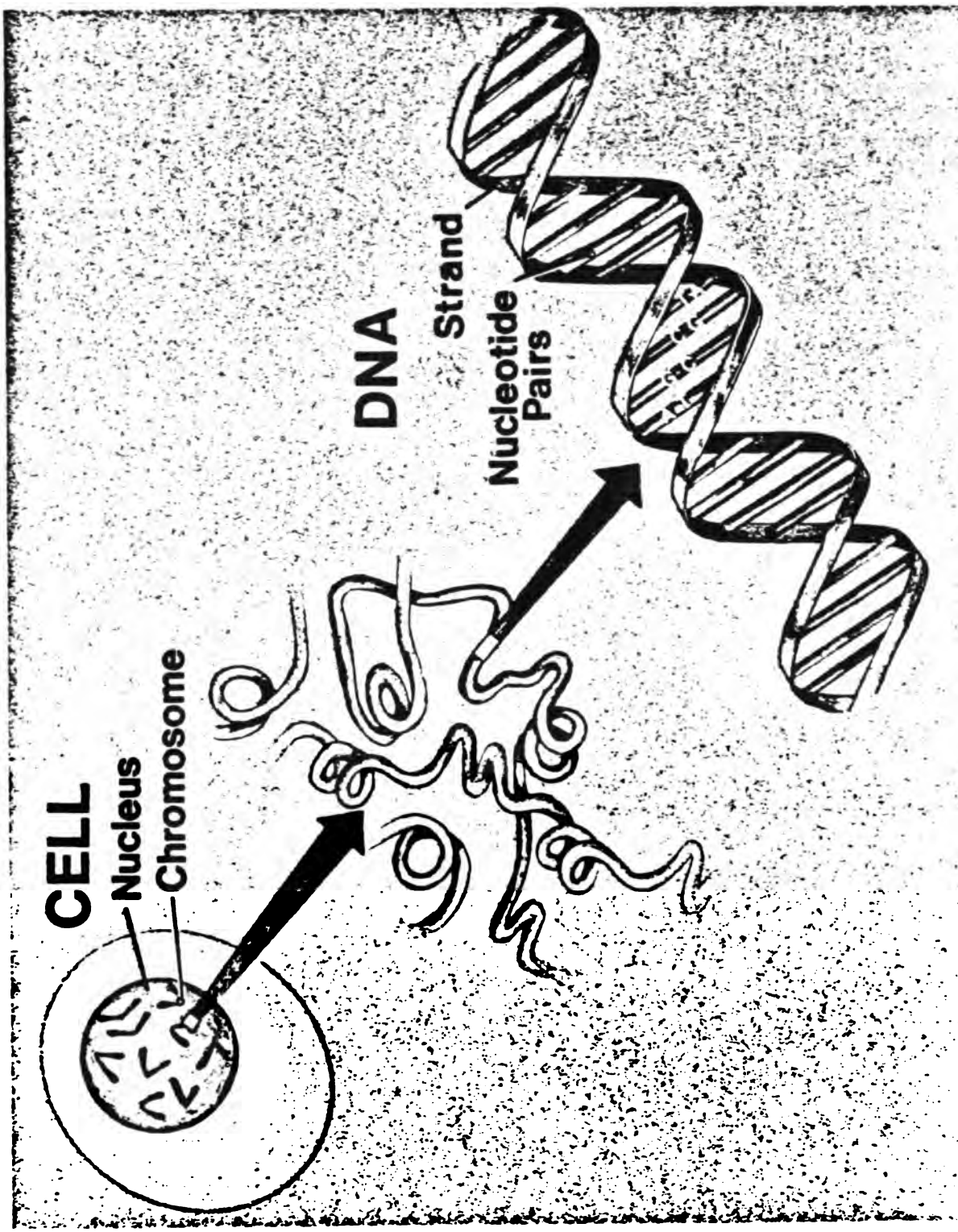

Fig. 1. The structure of DNA consists of two strands composed of four bases: Adenine (A), thymine (T), guanine (G), and cytosine (C). Th four bases are always paired on the DNA molecule in a specific way - A always with T and G always with C.

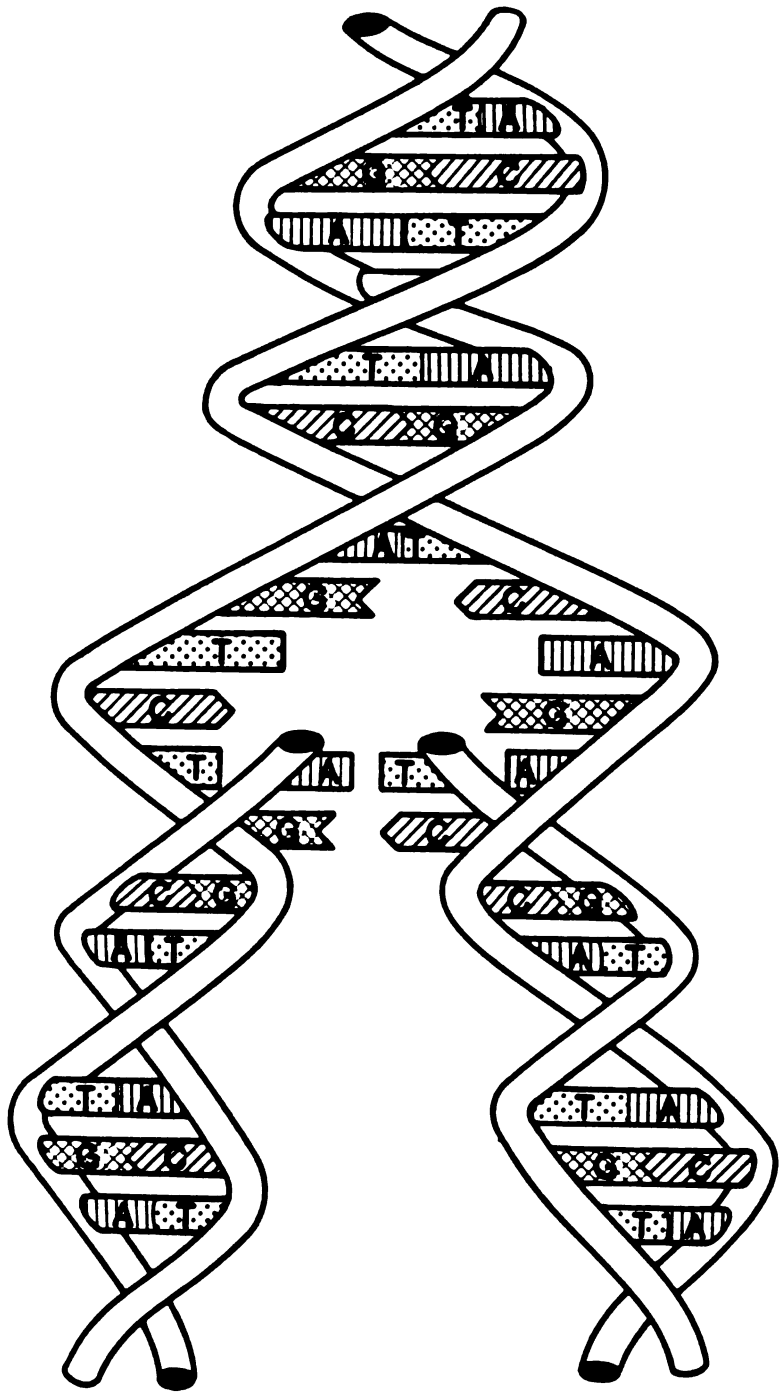

Figure 2. A model of DNA replication. As the two strands unwind, each serves as the template for generating a new strand.

This overview, which is admittedly superficial, focuses on (1) the diagnoses of infectious and genetic diseases by the use of restriction enzymes, DNA probes and monoclonal antibodies; (2) some applications of monoclonal antibody technology; (3) recombinant DNA (rDNA) vaccines, chemically synthesized vaccines, gene deletion vaccines and anti-idiotype vaccines; (4) some medically important proteins prepared by rDNA technology; and (5) novel procedures for the genetic improvement of livestock.

RECOMBINANT DNA TECHNOLOGY

In the early 1970's, Herbert Boyer of the University of California and Stanley Cohen of Stanford University isolated the DNA sequence that codes for human insulin. When that particular DNA sequence was transferred into E. coli, it turned the E. coli into "insulin factories". After Boyer's and Cohen's discovery, it was immediately apparent that recombinant DNA (DNA from two different organisms) might provide more effective and less expensive vaccines and pharmaceuticals. Time Magazine noted that "The whole affair left Wall Street slightly dazed".

Gene splicing or genetic engineering isn't all beer and skittles (Fig. 3). The first step in recombining DNA is to chop the donor DNA that codes for a virus, bacterium or a medically useful protein such as insulin into fragments. This is accomplished by using restriction endonucleases which cut the DNA at a specific nucleotide sequence.

The next step is to insert the donor DNA into a plasmid which transports the donor DNA into E. coli. Plasmids are doughnut shaped pieces of DNA found outside the chromosomes in bacteria. Plasmid DNA is cut with the same restriction enzyme and when mixed with the donor DNA, the ends of the donor DNA and the plasmid are "glued" with the ligase enzyme. Then the donor/plasmid recombinant is inserted into E. coli. The donor genes in the plasmid direct the production of a specific protein (viral, bacterial, or animal proteins for vaccines, insulin, growth hormones, etc). Thus, the E. coli is tricked into producing the desired donor protein which is placed in the E. coli by gene cloning. In 15 minutes at 37° C E. coli can make about 10,000 DNA copies. The new instructions are passed on to the next generation of E. coli.

Vectors are plasmids, or viruses used to transfer the "new" donor DNA into host cells such as bacteria, yeast, insect or animal cells.[20] Papovaviruses, papillomaviruses, herpesviruses, adenoviruses, retroviruses, bateriophages, insect viruses and many other viruses have been used as cloning and expression vectors.[83]

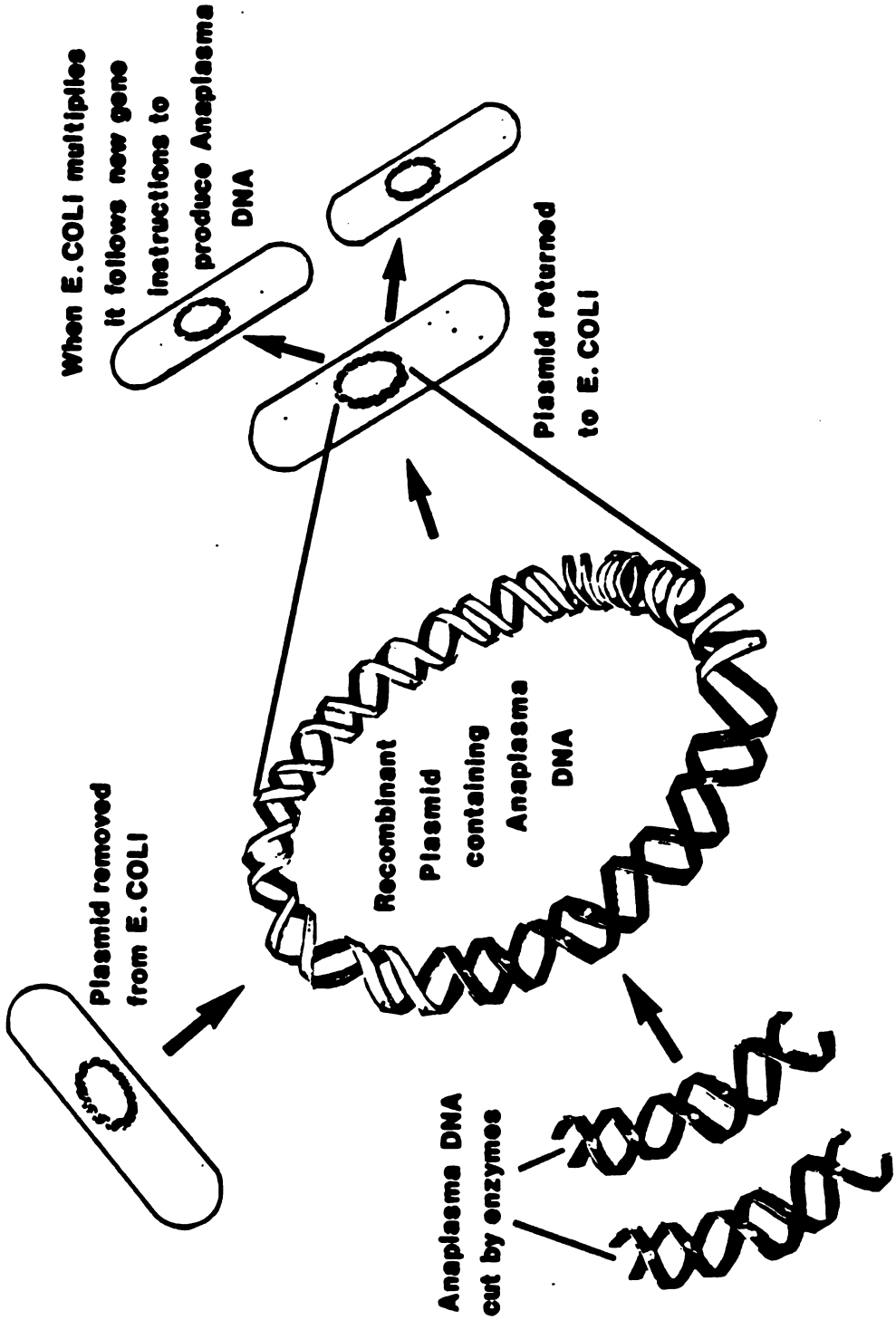

Fig. 3. Anaplasma DNA is used to illustrate the recombinant DNA procedure to clone Anaplasma DNA.

DIAGNOSIS

Diagnosis by Restriction Endonuclease Analysis (REA)

By the early seventies, Smith and others had isolated bacterial enzymes that chop up DNA at specific (restricted) sites.[115] The collected DNA fragments of disease agents can be separated by electrophoresis to map resulting restriction endonuclease fragments and determine differences or similarities in the genomes. Several recent investigations illustrate the advantages of REA in the identification and differential diagnosis of disease agents.

Whetstone and her coworkers investigated outbreaks of bovine herpes virus 1 (BHV-1), (IBR) by REA.[117] Their findings revealed that vaccine strains were probably the source of infection in 2 of 6 isolates collected from the field. In 4 additional isolates, the REA patterns were different from the vaccine viruses in their collection. Kennedy, et al compared suspected IBR isolates obtained from the mammary gland and a standard IBR strain by restriction endonucleases. They determined that the mammary gland isolates had restriction fragment profiles comparable to infectious pustular vulvo-vaginitis and not IBR.[52]

Restriction enzyme analysis coupled with monoclonal antibodies strongly suggest that canine parvovirus infecting dogs in the United States changed its antigenicity.[89] Following the first recognition of the disease in 1978, isolates were collected. Isolates studied after 1980 were antigenically different. Several explanations are possible (1) there was an antigenic drift of the virus due to immune pressure; (2) the new strain emerged in a vaccine; or (3) the new parvovirus strain was better adapted to growth in dogs.

Thiermann et al felt that the classification of leptospires based on microscopic agglutination was open to question. Their investigation using REA and DNA probes revealed a number of significant findings. While there were no detectable serologic differences, the reference strain hardjoprajitno, used in diagnostic tests and vaccines, differed in REA fragments at gene loci, thus allowing strain differentiation from North American hardjo-bovis strains.[63,112]

Holmberg and his coworkers[45] used restriction endonuclease fragments to conclude that antibiotic-resistant **Salmonella newport** of animal origin caused serious illness particularly in people on antibiotic therapy. Recently, Spika et al[106] analyzed DNA from **Salmonella newport** isolates from hospitalized and dead patients; hamburgers eaten by the same patients; dairy cows at abattoirs which were the source of the hamburger; and, dairies that sent the cows for slaughter.

They demonstrated antibiotic resistance to chloramphenicol which was conferred by a single unique plasmid (fragment) from the collected isolates.

Bovine isolates of **Mycobacterium paratuberculosis** were subjected to REA by Collins and coworkers.[21] They demonstrated that REA could not be used to type bovine strains of **M. paratuberculosis** because of close genetic similarity. However, they felt that REA might be employed to compare **M. paratuberculosis** strains isolated from goats and sheeps.

Diagnosis by DNA Probes

There is little doubt that DNA (gene) probes will, in a matter of a few years, revolutionize the diagnosis of infectious and genetic diseases and neoplasia in both humans and domestic animals.[53]

To make a probe, DNA is heated or treated chemically until the two strands separate. Each strand will recognize and bind to a strand of DNA that have complementary nucleotide bases. To put it another way, a DNA probe will "search" the tissues of an animal or an insect for the complementary nucleotide (sequence) of a pathogen. To determine whether binding (hybridization) has occurred, the single strand of the probe DNA is usually labeled with radioactive ^{32}P.

Denatured DNA is freed from clinical specimens (blood, saliva, urine exudates) and applied to nitrocellulose filters (dot-blot procedure). If the DNA sequences of the probe and the target DNA of the clinical specimen are complementary they will hybridize. Next, the filter is checked for the presence of the probe's label. The specimen is positive for the pathogen if the label is detected. If the specimen is negative, the labeled probe will not bind to the sample and will be washed away in the procedure. Although the "hot" radioactive probes are very sensitive, they have some disadvantages. The ^{32}P isotope has a half-life of only a couple of weeks and is a radiation hazard.

To facilitate commercial use of DNA probes, radioactive tags will have to be replaced with sensitive, long shelf-life non-radiolabeled tags.[12,62] Presently, most laboratories involved in making probes are utilizing the tenacious attraction of biotin and avidin from egg white. In this instance, the DNA probe is labeled with biotin and is detected by streptavidin, which is linked to horseradish peroxidase or alkaline phosphate which yield conspicuous color in the presence of their substrates and can be assayed. Also, the streptavidin can be conjugated with a fluorescent dye. Biotin-labeled probes have a long shelf-life and the assay time can be reduced to a couple of hours, whereas the radiolabeling procedure usually requires an

overnight radiograph. Some investigators using biotinylated probes have encountered sensitivity problems.

The observation that DNA and RNA can be detected in formalin fixed and paraffin embedded tissues was good news for pathologists. Thus, specific nucleic acids can be visualized on a microscopic slide by **in situ** hybridization.

There have been a few research reports, but currently no probes are commercially available for veterinary applications. The following papers concerned with animal pathogens provide some examples of probe research with various pathogens.

Gutekunst[43] detected latent viral DNA sequences in the trigeminal ganglia of swine which had recovered from pseudorabies. McFarlane et al have also employed a DNA probe to detect latent pseudorabies virus.[74] Interestingly, viral DNA was present in high concentrations in the spleen and liver of a seronegative pig that contained no detectable infective virus. These swine had been exposed to pigs latently infected with pseudorabies virus. Dorman et al[27] employed biotinylated DNA probes for the detection of bovine herpes virus 1 (BHV-1) DNA in cell cultures and nasal swabs and exudates from experimentally infected cattle. Dunn and coworkers also used a biotin-tagged probe to demonstrate BHV-1 DNA in infected cell cultures and nasal epithelial cells collected from inoculated calves. In addition to a new procedure for the diagnosis of BHV-1 infections, DNA probes will be worthwhile tool to study pathogenetic mechanisms and epidemiology.[28]

The diagnosis of subacute and chronic African Swine Fever Virus (ASF) always presents difficulties. Caballero and Tabares have detected ASF by DNA hybridization.[16] The probe will be used to elucidate the pathogenesis and epidemiology of the disease and will be of real value for the rapid diagnosis in swine and perhaps in pork products.

The definitive diagnosis of bluetongue virus infection and determination of serotypes in ruminants and insects can require 3-4 weeks using traditional virologic techniques. Indeed, the subject of bluetongue virus latency has caused some intellectual friction between livestock owners and disease control officials. Eventually, bluetongue probes currently being developed by at least two groups of investigators should provide a rapid, sensitive procedure for detecting bluetongue nucleic acids sequences directly from infected blood tissues and insect vectors.[1,97,107]

Davidson et al developed a dot-blot DNA hybridization test that detected Marek's disease virus in feather tips of infected chickens. The probe was both sensitive and specific.[24]

Goff and his coworkers constructed a DNA probe to detect **Anaplasma marginale** DNA.[42] This ^{32}P probe will impact future anaplasmosis epidemiologic investigations since it is species specific and will detect target DNA in as few as 250 infected RBC and individual infected ticks (Fig. 4).

Kingsbury reported a mycoplasma DNA probe that was intentionally prepared lacking species specificity so that it could be used for screening purposes. Kingsbury also pointed out that mycoplasma probes will help elucidate the role of mycoplasma in many diseases.[54]

Mainil and his coworkers employed probes to determine the prevalence of K99 (adhesion factor) and enterotoxins in **E. coli** isolates collected from cases of enteric and systemic disease.[71] Maddox and Wilson have also used DNA probes to detect enterotoxigenic **E. coli.**[70] DNA probes to identify **Salmonella spp** in meat and meat products have been reported by Fitts et al[32]. Such probes could also be used to trace the source of specific strains of bacteria in foodborne outbreaks. Probes have a distinct advantage over culturing in that they should be able to detect dead bacteria or bacteria in autolyzed tissues.

Probes will be offered as a replacement for serology and conventional antigen detection procedures. However, until they have a history of reliability, traditional methods of antigen detection and bacterial and viral isolation methods will remain as the "gold standard" of infectious disease diagnosis.

Although DNA probes to detect at least two human protozoal diseases (leishmaniasis and malaria) are being tested under field conditions, DNA probe research to detect protozoal diseases of livestock have been limited. McLaughlin et al[76] felt that probes may be used to detect babesia infected cattle and ticks, to identify strains, and relate virulence with particular strains of babesia.

McManus and Simpson reported that clones fragments of the ribosomal RNA gene of **Schistosoma mansoni** hybridize strongly to **Echinococcus** DNA. The authors feel that the technique is an excellent additional method for the identification and characterization of **E. granulosus** and **E. multilocularis.**[77]

DNA probes have been used to identify several human genetic diseases and somatic mutations associated with tumors. While these have been truly exciting advances, at this writing we have no probes that will detect an abnormal animal gene. For an example, almost 26% of the Arabian horses in the United States are estimated to be carriers of the gene for combined immunodeficiency.[92] Only test breedings of dams and sires can identify horses that are carriers of this autosoma recessive

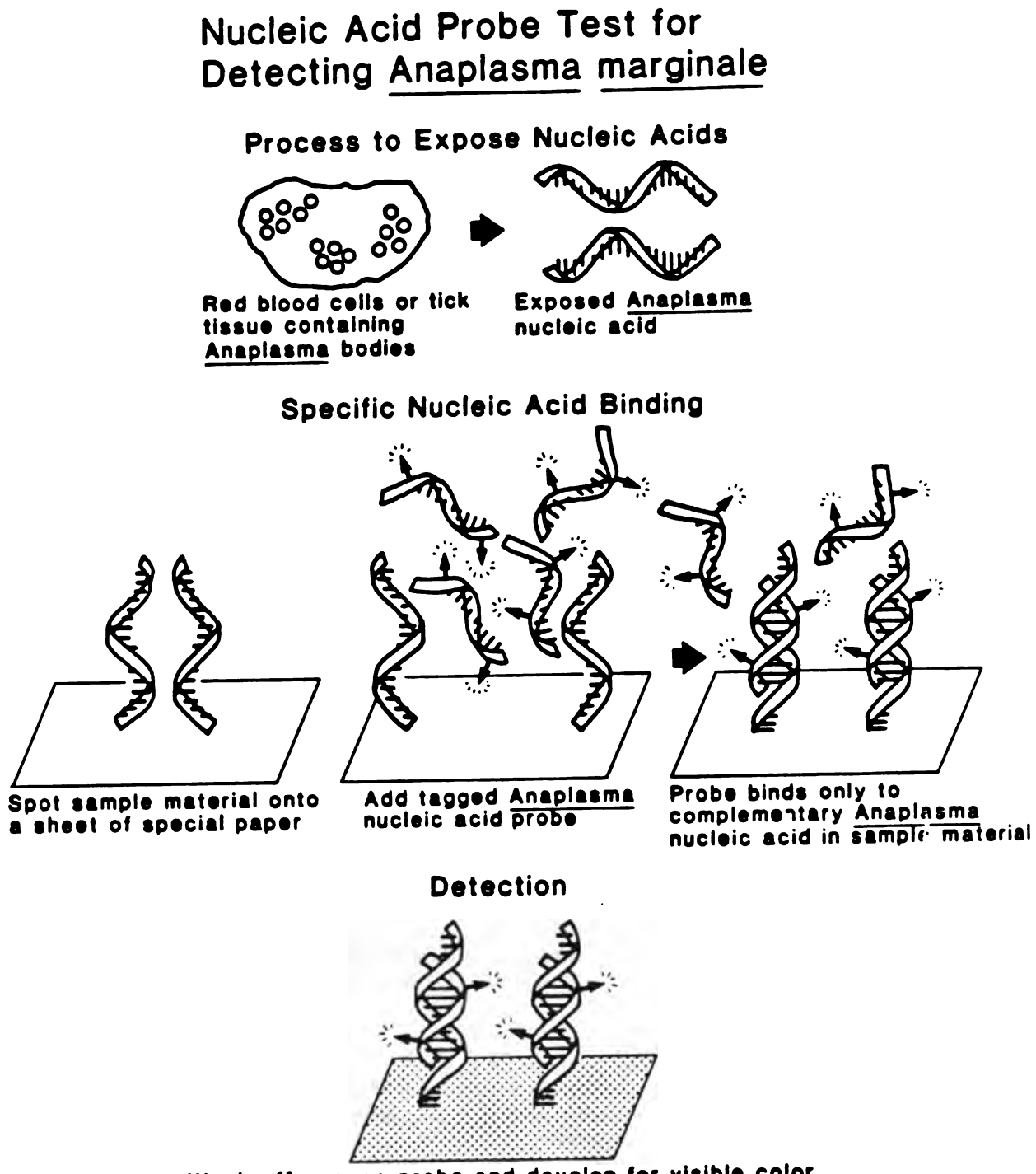

Fig. 4. An illustration shown the use of an Anaplasma DNA probe to detect infected RBC or ticks.

disease. A probe to detect the carriers of the abnormal gene would be of tremendous economic significance.

There are questions raised by the use of this technology.[39] How will their use be regulated? No doubt that within the next decade large livestock producers will have the capability to determine the infectious and genetic disease profile on their own farms. Then they will be able to ask their own computers for the options on how to handle the disease problem on their farms. One question that is certain to come up-- how will the diagnoses generated by these "on the farm" probes and monoclonal antibody kits be reported? Needless to say, the veterinary practitioner and disease control officials will be required to interpret the total health/disease picture on a farm, but biotechnology will arouse some thorny issues.

Monoclonal Antibodies (Mabs) for the Diagnosis, Prevention and Treatment of Disease

The employment of polyclonal antibodies at the turn of the century was a major advance in diagnostic medicine. The recent advent of Mabs has allowed significant advances in immuno-diagnostic medicine.[37,72,93,102]

The breakthrough occurred when Kohler and Milstein reported a mouse hybridoma cell that produced monoclonal or single antibodies[60] (Fig. 5). To obtain a hybridoma, a normal mouse is immunized with an antigen such as virus, bacterium, parasite or the cell membranes of a tumor cell. Later its spleen is removed and the B lymphocytes fused to continuously proliferating mouse myeloma cells. When fused into a hybridoma, the mouse myeloma cells take their coded instructions from the antigen-stimulated B cells of the immunized normal mouse. These fused cells called hybridomas are small "cellular factories" that divide continuously yielding progeny cells which continue to produce a single Mab. The hybridoma clones are screened to determine those that are secreting the desired monoclonal antibody. Then the appropriate hybridoma is propagated either in a mouse or cell cultures to produce large amounts of the required antibody.

Each Mab has a unique combining capacity in that it reacts with a single epitope. Monoclonal antibodies are widely used to study many basic and clinical problems. They have improved the sensitivity and specificity of present-day serological tests. Only a few examples will be given as Mabs have been the subject of vigorous research and several reviews.

Since the days of Pasteur, rabies isolates have been presumed to be antigenically homogeneous. Wistar Institute workers using Mabs reported differences in antigenic composition

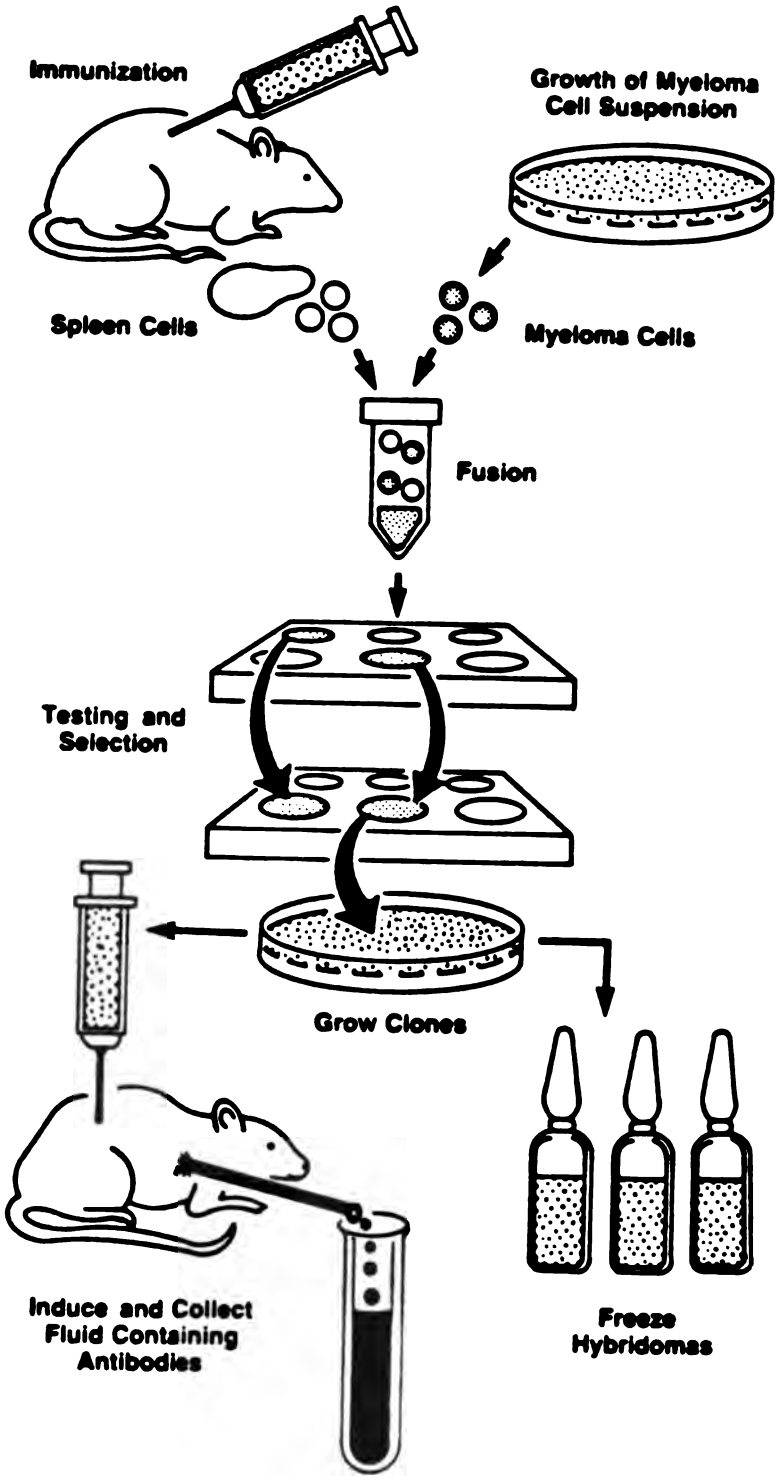

Fig. 5. A flow chart showing the production of monoclonal antibodies (ARS Drawing).

between different isolates of rabies or rabies related viruses obtained from various areas of the world.[33,34,118] Obviously, this information was important to rabies vaccine manufacturers.

Researchers have raised a Mab to enterotoxigenic **E. coli** (ETEC) K99 pili antigen to prevent attachment and colonization of ETEC.[101] If administered to calves orally within the first 12 hours postpartum, the severity of the diarrhea and death losses were reduced. Diagnostic kits employing Mabs have been useful in the identification and control of ETEC and other bacterial infections.

An extraordinary potential of Mab technology is the diagnosis and treatment of tumors. Experimentally Mabs laced with radioisotopes are employed to image tumors and determine whether metastases have occurred.

Probably the most enticing use of Mabs is in the therapy of tumors. Mabs can serve as a transport vehicle for cytotoxic materials to the tumor cell. Radioisotopes, diphteria, cholera, plant protein toxins and standard chemotherapeutic drugs have all been delivered directly to the target tumor cell. While these approaches are still in the experimental stage, it is a very active area of research.

Cohen[19] pointed out that protozoan and metazoan parasites are complex organisms involving a diversity of antigens. Mabs are being used to (1) isolate protective antigens; (2) improve immunotherapy; (3) determine antigens involved in morphogenesis; (4) serologically differentiate parasites; (5) analyze mechanisms of acquired immunity; (6) study immunopathology mechanisms and (7) improve current serodiagnostic tests.

In non-infectious diseases such as those of the endocrine system, Mabs can be used to measure the concentration of hormones, body metabolites or other substances of interest.

There really should be no argument as to whether Mabs or DNA probes will be the diagnostic method of choice because both procedures have a number of applications and there are some situations where one procedure will be preferred over the other. The two techniques will compete for reliability, accuracy, speed , cost and simplicity. DNA probes are particularly appropriate for the diagnosis of genetic disease changes and deletions in gene sequences are responsible for diseases having a genetic origin.

"HIGH TECH" VACCINES

One of the greatest impacts of biotechnology will be in the manufacture of vaccines. Experimentation is in its infancy

and there are plenty of growing pains. The development of genetically engineered vaccines is very expensive. Most veterinary vaccines are relatively inexpensive, so that there is little incentive to manufacture a genetically engineered product unless there are difficulties with current vaccines or if there are diseases for which an effective vaccine cannot be readily produced by conventional means.

Only a few examples of new methods of vaccine preparation for domestic animals will be given. For a more detailed treatment of the subject readers can consult recently published reviews.[2,3,14,17,18,46,55,85]

Recombinant DNA Vaccines

Bachrach and his coworkers at Plum Island National Animal Disease Laboratory and workers at Genentech cloned a DNA sequence (VP-1) coding for a major immunogenic surface protein of foot-and-mouth disease. The gene was expressed in **E. coli** producing a protein that evoked high levels of neutralizing antibody and protection against challenge with a homologous strain of foot-and-mouth disease virus.[2,3,61,81]

Two groups of Australian workers[29,111] have expressed pili genes of **Bacteroides nodosus** in **Pseudomonas aeruginosa.** They concluded that the recombinant DNA derived vaccine was as effective as vaccines prepared from native pili. Interestingly, the vaccine had value in treatment regimens. Lehr et al also employed **Pseudomonas aeruginosa** as an expression vector for **Moraxella bovia** pili genes.[64] Their recombinant vaccine offers promise for the eventual control of pink eye. Genetically engineered vaccine directed against the somatic pili of enterotoxigenic **E. coli** was probably the first "high tech" vaccine to become commercially available.[44]

Because of the numerous antigens encountered in larger parasites, research directed to cloning and expressing appropriate genes for candidate vaccines has been difficult.[4,10,22,23,36,75,119]

Investigators using vaccinia vectored vaccines have had considerable success in evoking high levels of neutralizing antibody and in some instances protection against human pathogens, including herpes viruses, hepatitis B, influenza, Epstein-Barr virus and rabies.

More recently, veterinary researchers have employed vaccinia virus recombinants as experimental vaccines: vesicular stomatitis (Mackett et al[69]), fowl pox (Boyle and Coupar[11]), and Sindbis virus (Franke et al[35]), (Wedman and coworkers[116]). Gillespie et al[38] studied the response of dairy calves to vaccinia vectored human hepatitis B surface antigen and herpes

simplex virus, type 1 glycoprotein D. Calves developed neutralizing antibody to vaccinia virus and herpes simplex glycoprotein but not to hepatitis B surface antigen. Since there is a possibility of using other pox viruses as vectors, Baxby et al have discussed their maintenance in nature.[6]

The use of vaccinia vectored rabies vaccine administered by the oral route offers real promise for the control of rabies in wildlife. Raccoons fed vaccinia/rabies recombinant virus developed rabies neutralizing antibody and were refractory to rabies challenge 205 days after the feeding.[98]

Two steps are required to prepare hybrid vaccinia virus vaccine. First, a plasmid as described previously is constructed containing the DNA of the donor virus. In the second step, the viral donor DNA segment in the plasmid is inserted into the vaccinia virus genome. The recombinant vaccinia is grown in cell culture producing the foreign viral immunizing protein of interest. There is room for a great deal of foreign DNA in the large vaccinia genome so that it is possible to construct a hybrid recombinant vaccine against several diseases.[90]

If desired, vaccinia vectored vaccines could be administered by the intranasal route. The Chinese immunized themselves centuries ago by blowing attenuated smallpox scabs through a bamboo tube into the nostrils of vaccinates.

The reintroduction of vaccinia virus as a vector has been criticized. Murphy[84] and Quinnan[95] have discussed the special safety concerns of vaccinia vectored vaccines for immunoprophylaxis against animal diseases.

Other pox virus are being used as experimental vectors to control rabies in wildlife. Also, poultry researchers feel that fowl pox could be used as vector in vaccines prepared against Marek's disease, Newcastle disease, infectious bronchitis, infectious bursal disease and fowl pox if it was used as a vector.

Synthetic Vaccines

Workers at Scripps Clinic and Research Foundation have employed procedures that are more direct than recombinant DNA techniques.[7,8,9] First, the essential protein is identified that stimulates the animal to make antibodies against a particular pathogen. Then individual amino acids that make up the protein are linked in the right order to produce a synthetic protein vaccine. It is important to get the proper amino acids folded in the correct configuration for the vaccine to produce effective immunity.

A synthetic protein vaccine offers several potential advantages.[103] Because a synthetic vaccine contains amino acids that mimic only the antigenic portion of the immunogen, there is no way the synthetic vaccine could mutate to virulence or become so "overattenuated" that it does not immunize. In addition, because cell cultures, chicken embryos, or other animal tissues are not used in the preparation of the vaccine, the prospect of a synthetic vaccine carrying an incompletely inactivated or latent virus is not possible.

Bittle and his coworkers,[7,8,9] chemically synthesized peptides corresponding to two different regions of the VP-I polypeptide of foot-and-mouth disease virus and induced neutralizing antibody in cattle, guinea pigs, and rabbits. One injection of a single peptide protected guinea pigs against a later challenge with pathogenic virus. Shinnick et al has commented on two critical factors in the development of synthetic vaccines: (1) selection of the most appropriate (protein); and
(2) the choice of a proper adjuvant.[103]

It is unlikely that any synthetic protein vaccine will be successful unless a potent adjuvant is employed to maximize the immune response. It is a key issue because there is a dearth of acceptable adjuvants. There is currently a sizeable effort to develop safe and effective adjuvants. There is a great deal of interest in an adjuvant or what has been termed an immunostimulating complex (ISCOM).[82,87] This adjuvant is only suitable for sub-unit virus vaccines and not intact whole virus vaccines.

Gene Deletion Vaccines

For many years virologists have wondered if viral genes coding for virulence could be removed from a live virus vaccine. Recently, Saul Kit and his coworkers reduced the virulence of the BUK vaccines strain of pseudorabies by engineering a mutation into the thymidine kinase (TK) gene so that the vaccine virus had no detectable TK activity.[56,59] Without thymidine kinase the virus cannot multiply in the central nervous system of pigs and establish latency. Thousands of doses of the TK-(minus) pseudorabies vaccine have been used with no reported difficulties. The TK gene has also been deleted from infectious bovine rhinotracheitis (BHV-1) vaccine strain which would allow similar development of a cattle vaccine.[57,58]

An environmental assessment paper prepared by the Veterinary Services, Animal and Plant Health Inspection Service, U.S.D.A. (April 29, 1987) revealed a double gene deletion

vaccine for pseudorabies vaccine. In addition to a thymidine kinase deletion, a second deletion removed the gene coding for viral glycoprotein which prevents antibodies being evoked to this glycoprotein. This second deletion allows vaccinated pigs to be identified from pigs naturally infected.

A recent APHIS environmental assessment (Aug 17, 1987) reported a pseudorabies vaccine having a triple gene deletion with a marker. In addition to the TK and viral glycoprotein gene deletions, SyntroVet virologists have produced a deletion in the internal and terminal repeat regions of the pseudorabies vaccine strain which reduced the virulence of the strain for pigs. They have also added a lactase marker gene which identifies pigs vaccinated with the SyntroVet vaccine from those pigs naturally infected or those vaccinated with other vaccines.

Anti-Idiotype Vaccines

An idiotype is the site on an antibody molecule that binds with a foreign antigen. Since the site itself can act as a foreign antigen, a monoclonal antibody can be raised against the idiotype that will have a mirror image conformation which mimics the original foreign antigen. This anti-idiotypic antibody can act as a surrogate vaccine to induce active immunity.

Anti-idiotype vaccines have some advantages: (1) they are no infectious; (2) large amounts of monoclonal anti-idiotypic antibodies can be prepared; (3) the antigenic determinants of some parasites are carbohydrates and cannot be genetically engineered and (4) an anti-idiotypic vaccine might be prepared that mimics an antigenic determinant that is common to many pathogen strains. The use of anti-idiotypes as vaccines have been recently reviewed.[15,73] As with any new technology, there will be a multitude of problems but some believe that anti-idiotypes will be the "vaccines of the future".

MEDICALLY IMPORTANT PROTEINS

The dramatic discoveries in biotechnology involve the recombining of DNA in bacteria. Most of the early work was accomplished using E. coli as the host organism, however, it is now possible to employ yeast and cultured cells from higher organisms as hosts.

Modern day biotechnology received its big impetus in 1978 when insulin was produced by rDNA procedures.[41] Because of the well recognized side effects of animal source insulin, rDNA insulin is destined to be the insulin for future needs.

Genentech's engineering of human growth hormone (hGH) in 1979 was another remarkable achievement.[40,47] This hormone which is used to treat pituitary dwarfism was previously only available by extracting human pituitary glands from cadavers. The demand for hGH was always greater than the supply, and the use of virus contaminated pituitary origin hGH caused Creutzfeldt-Jacob-disease a slow and always fatal human virus disease.

The effects of growth hormone on domestic animals have intrigued scientists for decades. However, because of the limited availability of natural growth hormones, research efforts were curtailed. The situation dramatically changed following the production of bovine growth hormone (bGH) by genetic engineering. Recombinant bovine growth hormone (bovine somatotropin) is identical to bGH. Bauman et al[5] reported that long-term treatment with recombinant bGH increased the milk yield by 20-40%. Smith and Bauman have recently summarized the response of cows to bGH and discussed the role of bGH in future dairy production.[105]

This research is not without controversy. On April 1, 1986 Jeremy Rifkin (Counsel for the Foundation on Economic Trends); the Humane Society of the United States and other groups petitioned the Food and Drug Administration to deny approval of any new drug application for the use of bovine growth hormone in dairy cows to stimulate milk production.

In an Associated Press report (May 1, 1985) R.J. Kalter of Cornell University said "bGH will be a mixed blessing". He estimated that the national dairy herd could drop 30% from 11.2 millions cows to 8 million if bGH is used extensively. Kalter further warned that increases in milk production through the use of bovine growth hormone will result in a fall in milk prices and the number of dairy cows and dairy farms will have to decline substantially to restore market equilibrium. Kalter pointed out that "few policy makers are considering the broad impact of biotechnology, and little research in this area is underway."[51]

Porcine pituitary derived (pGH) has a marked effect on the rate and efficiency of growth in the young pig. Etherton and his coworkers reported that growth rate was increased and carcass fat was decreased and muscle mass increased.[30] The pigs were treated daily for 35 days. Steele et al[109,110] have had similar findings using porcine pituitary derived pGH. While genetically engineered pGH will have a tremendous future impact on the pork industry, a delivery system such as an implant that releases the daily dose of pGH will be required.

Baculoviruses, are insect viruses, that can be propagated in insect cell culturees, such as the fall army worm. Foreign

genes inserted into the baculovirus have expressed human beta-interferon, C-MYC protein, interleukin 2, bacterial beta galactocidase and human alpha interferon[83]. The baculovirus vector system may provide a relatively inexpensive way of mass producing genetically engineered proteins.

The technology has opened the way for novel possibilities for a variety of medical applications. Some additional examples are the interferons, interleukins and other lymphokines, human and animal hormones, enzymes, gene preparations, amino acids, blood proteins and antihemophilic factors, specifically factors VIII and IX used for the therapy of hemophiliacs.[86]

GENETIC IMPROVEMENT OF LIVESTOCK

Molecular Basis of Disease

A basic question has always been: Why do certain animals get diseases while others do not? Geneticists have tried to link genetic traits with disease susceptibility without a great deal of success. Until recently, they simply have not had the tools.

Although ample evidence has been obtained which shows variations in immune response are under genetic control, it has been difficult to develop simple methods of assay that can be used to genetically type humans and animals and correlate the patterns of inheritance of disease resistance with known genetic traits. The advent of monoclonal antibody and recombinant DNA technology has provided the means to surmount this problem and has already yielded information on one gene system that plays a major role in regulating the immune response.

Both in humans and in animals, the major histocompatibility complex (MHC), a cluster of genes that an animal inherits from its parents, are clearly associated with susceptibility and resistance to disease.[91,104] Immune response genes determine or limit the host response to bacteria, viruses, other pathogens and foreign antigens. Lunney[66,68] and Davis et al[26] have reviewed the genetic control of host resistance to several diseases.

Variations in the gene products of the MHC correlate with variations in the capacity of the individual's immune system to mount an effective response to native pathogens and to vaccines. Studies are now underway to characterize the MHC in domestic animals using monoclonal antibodies.[25] The application of this technology will improve our ability to identify and breed resistant animals.

Lunney and Murrell[67] using swine lymphocyte antigens have studied the genetic resistance of swine to **Trichinella spirallis** infection. Their preliminary findings revealed that one inbred line of miniature swine (SLA c/c haplotype) had a lower burden of **T. spiralis** larvae in the tongue following challenge than other inbred swine. The lower muscle burden correlated with early humoral antibody response.

Important information has been gained in dairy cattle which shows that disease progression in animals infected with bovine leukemia virus is associated with genetic variation in the MHC.[65] The findings emphasize the potential afforded the food animal industry by new advances in technology.

Sex preselection in sperm and embryos

If the sex of domestic animals could be predetermined, it would allow more rapid genetic and increased production efficiency, and increased management flexibility. Along with artificial insemination and embryo transfer, the sexing of sperm or embryos would be very useful in animal production.

Johnson and his coworkers[49] have used flow cytometry to analyze and separate sperm cells by measuring small differences in the DNA content. The female (x) chromosome has more DNA than the male Y chromosome. In all samples, the sperm sex ratio was 50:50.
Because the sperm cells lose their tails during the sorting process, the method cannot be used in a breeding program. However, information gained from these experiments is laying the groundwork for future practical systems.

Johnson[48] has reviewed the procedures that have been reported to sex embryos including chromosomal analysis assay for X linked enzyme, immunologic methods to detect H-Y antigen on male embryos and DNA probes specific for Y chromosomes. But Johnson points out that no current sorting procedure is sufficiently accurate, rapid, simple, and without side effects on the sperm to be used on a practical basis.

Transgenic Animals (The Addition of Genetic Information to Embryos)

Man has been improving the genetic quality and productivity of livestock for thousands of years. Classical breeding trials, albeit painfully slow, have served us well. But improvement necessarily has been based on the selection of genes already present in the population.

In the future, clasical breeding methods may be bypassed by injecting genes coding for desirable traits directly into the

pronuclei of one-cell embryos so that the introduced genes divide each time the cells divide. The resulting animal is termed transgenic.

In 1982, pivotal research appeared that was hailed by the scientific community, astounded the general public, and incurred the wrath of activists who charged that the research violated "the integrity of the species". Brinster and Palmitter[88] microinjected the gene for rat growth hormone into mouse embryos and implanted them into foster mothers. Some of the mice carrying the introduced gene grew faster, and weighed 70-80% more than their litter mates that didn' have the gene.

That the success of this trial would direct future research efforts in domestic animals was obvious.[13] Pursel and his coworkers[94] have recently documented their own gene transfer investigations and those of others. They reported that transgenic pigs with elevated plasma hGH or bGH did not grow faster than little mates, but they were dramatically leaner and significantly more efficient in feed utilization. The transgenic pigs weighed 20% less, had reduced appetite and a few had persistent diarrhea. The meat and milk and other animal products of transgenic animals would have to be monitored especially in the instance of transgenic animals receiving growth hormone genes.[50]

Australian investigators[79] have inserted human growth hormone genes into sheep, pigs, goats, and cattle in hopes of increasing wool growth and milk yield. They now have first generation animals and are characterizing the gene inheritance and expression (Genetic Engineering News, May 1987).

According to Genetic Engineering News (Sept. 1987) Chinese workers have succesfully introduced rat growth hormon genes into carp and claim that some transgenic fish grew to twice their normal size.

In addition to stimulating more rapid growth, gene transfer of immune response genes to confer disease resistance is an intriguing possibility. While the potential uses of transgenic animals is exciting, the regulation of the introduced genes needs careful study.

Salter and his coworkers[99] have developed techniques for insertion of avian retroviral genetic information into the chicken germ line. They hope to determine how these fragments of foreign proviral DNA express themselves and affect the perfomance of the chicken. Possibly a retrovirus might be employed as a vector to incorporate desirable genes for such traits as growth, egg production, disease resistance and producing a "super chicken".

A very provocative article in the Genetic Engineering News, Oct. 1987, may foretell the direction of future transgenic research. Researchers at the Institute of Animal Physiology and Genetics in Edinburg showed that therapeutic proteins (human factor IX - an antihemophilic substance) can be obtained from the milk of transgenic sheep.[80]

Cloning Embryos

Embryos can be divided microsurgically resulting in identical twins. Indeed, thousands of calves have been produced from split embryos. While, more than four identical copies could theoretically be produced, Seidel has pointed out that cloning procedures do not work with advanced embryonic cells. At the present time the only practical method for cloning is dividing embryos.[100]

Sheep/Goat Chimeras

Researchers have tried to cross sheep with goats for sometime but the pregnancies never progressed past 60 days. Recently scientists in England and West Germany have succesfully combined embryonic sheep and goat cells and transplanted the resultant combination embryo into a surrogate sheep or goat dam.[31,78] Some of the progeny were apparently normal along with some "patchwork" appearing offspring that the press called "Geeps". Six of the sheep/goat hybrids (chimeras) looked like lambs but had patches of goat hair. Other hybrids had the appearance of goat kids but they had patches of sheep wool. One hybrid behaved like a male goat but was infertile.

Although these experiments have stimulated some stormy sessions between activists and scientists, procedures resulting in the production of chimeras will aid in the basic investigation of cell differentiation and cellular interactions in embryonic stages. However, there are some immediate practical applications. It should make it easier to rear embryos of endangered species in the uterus of other animals. Indeed, zoo veterinarians succesfully tranferred a zebra embryo into the uterus of a quarter horse. Perhaps a valuable hybrid such as a mule could be produced using chimera procedures.

The U.S. Patent Office has recently ruled that new animals altered or mutated by genetic engineering or other scientific techniques are patentable. The new ruling allows protection for man-made animals no matter how they are made. According to Internal Medicine World Report (Sept. 1987) there are about 15 patent applications awaiting approval by the patent office.

Summary

Genetic engineering technology is advancing at almost an inconceivable rate and there are many potential applications but few practical uses exists today.

While I have focused on biotechnology, our ability to study and manipulate DNA has changed our approach to disease research. DNA technology now allows us to define pathogenesis at the molecular level.

A scientist is always at the mercy of his tools. With recombinant DNA, restriction enzymes, DNA probes, monoclonal antibodies and the micromanipulation of embryos, we are entering into a decade of spectacular basic and applied research that will have a profound impact on our livestock industry.

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GENE TRANSFER IN CROP IMPROVEMENT*

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Transfer of genes between plant species has played an important role in crop improvement for many decades. Useful traits such as resistance to disease, insects, and stress have been transferred to crop varieties from noncultivated plants. Recombinant DNA methods greatly extend (even outside the plant kingdom) the sources from which genetic information can be obtained for crop improvement. Gene transfer systems based on recombinant DNA are available for several crop species and are under development for others. The concerted use of traditional and more recent methods for plant genetic manipulation will contribute to crop improvement.

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Rapid Progress is being made in developing the tools for manipulating genetic information in plants by recombinant DNA methods. Plant genes are being cloned, genetic regulatory signals deciphered, and genes transferred from entirely unrelated organisms (notably bacteria and a virus) to confer new agriculturally useful traits on crop plants. Recombinant DNA methods significantly increase the gene pool accessible for crop improvement.

In this review we summarize and illustrate with selected examples the long history of gene transfer by plant breeders between plant species and even between plants from different genera. We describe the use of recombinant DNA-based methods for gene transfer to plants and indicate with examples how these may contribute to the future of crop improvement. Our analysis highlights the important role continuing development of technology (Fig. 1) has played in expanding the range of organisms from which genetic information can be mobilized to plants. We conclude with some views on issues related to the use of technology in crop improvement and the future strength of agriculture.

Gene Transfer Through Hybridization

Plant breeding and intraspecific gene transfer. Plant breeding as a science began in the 19th century with discoveries of how plant traits are inherited(1). The early years saw transfer and reassortment of large numbers of genes in heterogeneous cultivated populations (landraces). Breeders steadily expanded their search for new genetic variation to the entire crop species, including noncultivated populations. These were gene transfers within the species. It is from such exchanges that our modern cultivated varieties originated (2). Often, however, the crop species does not contain sufficient genetic diversity to allow the desired improvements. The search for added diversity has been a stimulus for plant breeders to adopt new technology.

In simple terms, plant breeding is the selection of plants with desired traits after the sexual exchange of genes by cross-fertilization between two parents. When one parent is a cultivated variety and the other a wild relative, an improved variety is formed by back-crossing to the cultivated parent and selecting for the desired combinations of characteristics. Plant breeding has developed into a sophisticated science, aided in part by the application of statistical tools. The alliance of genetics with probability theory has allowed plant geneticists to arrive at more efficient models for the combination and selection of genes in populations and breeding lines. Statistical methods are now indispensable in the design of field experiments and in the prediction and analysis of results (1).

The definition of a plant species rests on the concept of genetic isolation. Nevertheless, sexual exchange of genes between species can and does occur in nature without human intervention. One of the better documented cases of such transfer is that between maize (**Zea mays**) and teosinte (**Z. mexicana**) (3). Use by plant breeders of sexual exchanges between species as sources of genetic variability to improve crops has been made possible during the past 80 years by the discovery of efficient ways to circumvent the natural barriers to genetic exchange by sexual mechanisms.

Interspecific gene transfer. For certain crops, plant breeders in the 20th century have increasingly used interspecific hybridization for the transfer of genes from a noncultivated plant species to a crop variety in a related species (Table 1). The exploitation of interspecific hybridization for crop improvement is illustrated by the advances made in wheat breeding during this century. Gene transfer from related species into cultivated wheat began in 1930. Mc Fadden (4) transferred resistances to stem rust and loose smut diseases from tetraploid emmer (**triticum tauschii**) to hexaploid bread wheat (**T. aestivum**). The resulting bread wheat variety, "Hope", was widely grown in the United States and was responsible for one of the longest rust-free periods in the history of U.S. wheat cultivation. Other genes for resistances to races of stem rust and powdery mildew and to Hessian fly have since been incorporated from **T. timopheevi, T. monococcum,** and **T. turgidum** into a number of bread wheat varieties (4).

Another early example of gene transfer to a cultivated crop species by interspecific hybridization is in tomato. In 1936, Tucker and Bohn transferred a gene conferring resistance to race 1 of the fusarium wilt fungus from weedy **Lycopersicon pimpinellifolium** to the cultivated tomato (**L. esculentum**) (5). Because occurrence of this pathogen is global, resistance to race 1 conferred by the **L. pimpinellifolium** gene is considered essential in commercial tomato production throughout the world (5). More recent applications of interspecific gene transfer include successful wide hybridization between the cultivated soybean (**Glycine max**) and its wild perennial relatives (6).

Intergenic gene transfer. Successful interspecific transfer of traits from wild species to domesticated relatives in the same genus was a precedent for attempts at even wider crosses, including those between members of different genera. A growing understanding of the origins of our crop species was also a factor. There is evidence that some of our modern crop species, such as rapeseed (**Brassica napus**), tobacco (**Nicotiana tabacum**), and wheat, originated in nature by hybridization between different species or genera. The available evidence indicates, for example, that the ancestor of **B. napus** was a

hybrid between B. oleracea and B. campestris. The creation of a new plant species has been mimicked in the modern era by the intentional hybridization of species from the genera Secale (rye) and Triticum (wheat) to create a new cereal crop, Triticosecale (triticale) (7).

Intentional crosses between species in different genera have also successfully transferred specific traits into crop species (Table 1). Here also some of the better documented examples come from the annals of wheat breeding. Hybridization between cultivated wheat and species of wild grasses from the genera Aegilops, Agropyron and Secale has been used to transfer various traits, including salt tolerance and disease resistance, into the crop (8). Advances in intergeneric gene transfer continue today. For example, the transfer of traits of cold tolerance, insect tolerance, and disease resistance from Solanum lycopersicoides to cultivated tomato by intergeneric hybridization may now be possible because Rick and his co-workers have succeeded in obtaining sesquidiploid hybrids between S. lycopersicoides and cultivated tomato (9).

Methods for production of hybrids. Natural barriers to interspecific and intergeneric hybridization make creating such hybrids difficult. Successful gene transfers by these methods begin with pollination of the flowers of one of the two species with pollen from the other. The gametes of the two species unite, and successive cell divisions produce an embryo. Development of the embryo and the endosperm associated with it gives rise to a mature seed, which upon germination produces a hybrid plant. The resulting complement of chromosomes must be stable so that the hybrid is fertile. Death or sterility can occur because of failure at any of the many steps in the process leading to a hybrid plant.

Even if an interspecific cross produces a viable zygote, incompatible genic interactions can prevent normal embryo or endosperm development. In such situations, the embryo may not survive. Organ culture techniques pioneered in the 1930s have been used to culture isolated embryos. The conditions used are designed to supply the life support for the hybrid embryo that is normally supplied by maternal tissue and the endosperm in early stages of embryo development and by the cotyledons (or in cereals the endosperm) during germination (10). Rescue of embryos in culture was a key tool used in obtaining the sesquidiploid of tamoto and S. lycopersicoides (9).

The youngest immature embryos that can be cultured in vitro generally are those that show observable signs of differentiation. Treatment of the ovule or seed with plant hormones may allow development of the embryo within the

Table 1

Examples of agriculturally important genes and traits transferred to crop plants by interspecific or intergenic hybridization. Though selective, the examples given are representative of the plant families in which such transfers have been most successful. The two families dominating the list are the Gramineae (wheats, oat, rice, and maize), and the nightshade family, Solanaceae (tomato, potato, and tobacco). TMV, tobacco mosaic virus.

Crop species	Donor species	Trait
Avena sativa (oat)	**A. sterilis**	Increase yield 25-30%
Beta vulgaris (sugarbeet)	**B. procumbens**	Sugarbeet nematode resistance
Brassica napus (swede turnip)	**B. campestris**	Clubroot resistance
Cucurbita pepo (pumpkin)	**C. lundelliana**	Mildew resistance
Gossypium hirsutum (cotton)	**G. tomentosum**	Nectariless (decreased incidence of boll rot)
Gossypium hirsutum	**G. raimondii**	Rust resistance
Lycopersicon esculentum (tomato)	**L. hirsutum**	Bacterial kanker resistance
Lycopersicon esculentum	**L. peruvianum**	Nematode resistance
Lycopersicon esculentum	**L. peruvianum**	Jointless (facilitates clean fruit harvest without stems)
Lycopersicon esculentum	**L. peruvianum**	TMV resistance
Lycopersicon esculentum	**L.pimpinelli-folium**	Fusarium wilt race 1 resistance
Nicotiana tabacum (tobacco)	**N. glutinosa**	TMV resistance
Nicotiana tabacum	**N. longiflora**	Blackfire resistance
Oryza sativa (rice)	**O. nivora**	Grassy stunt virus resistance
Ribes nigrum (black currant)	**R. sanguineum**	Mildew resistance
Ribes nigrum	**R. grossularium**	Gall mite resistance
Solanum tuberosum (potato)	**S. acaule**	Potato virus X resistance
Solanum tuberosum	**S. demissum**	Late blight resistance leaf roll resistance, potato virus Y resist.
Solanum tuberosum	**S. stoloniferum**	Late blight field resistance, potato virus A resistance, potato virus Y resistance

Crop species	Donor species	Trait
Triticum aestivum (bread wheat)	Aegilops comosa	Stripe rust resistance
Triticum aestivum	Aegilops ovata	High kernel protein
Triticum aestivum	Aegilops speltoides	Stem rust resistance
Triticum aestivum	Aegilops squarrosa	Leaf rust resistance
Triticum aestivum	Aegilops umbellulata	Leaf rust resistance
Triticum aestivum	Agropyron elongatum	Leaf rust resistance, drought tolerance
Triticum aestivum	Secale cereale	Yellow rust resistance powdery mildew resistance, winter hardiness, leaf rust resistance, stem rust resistance
Triticum aestivum	T. monococcum	Stem rust resistance
Triticum aestivum	T. timophevii	Stem rust resistance
Triticum durum (durum wheat)	T. monococcum	Stem rust resistance
Zea mays (maize)	Tripsacum dactyloides	Northern corn leaf blight resistance

incompatible ovule until a stage is reached at which the embryo can be cultured in vitro. For example, gibberellic acid treatment of an immature wheat kernel bearing a wheat-barley hybrid embryo will keep the embryo alive until it is approximately 10 days old, at which time it can be removed and cultured (11). When the cultured embryo has fully differentiated, it can then be transferred to a suitable medium for growth and development.

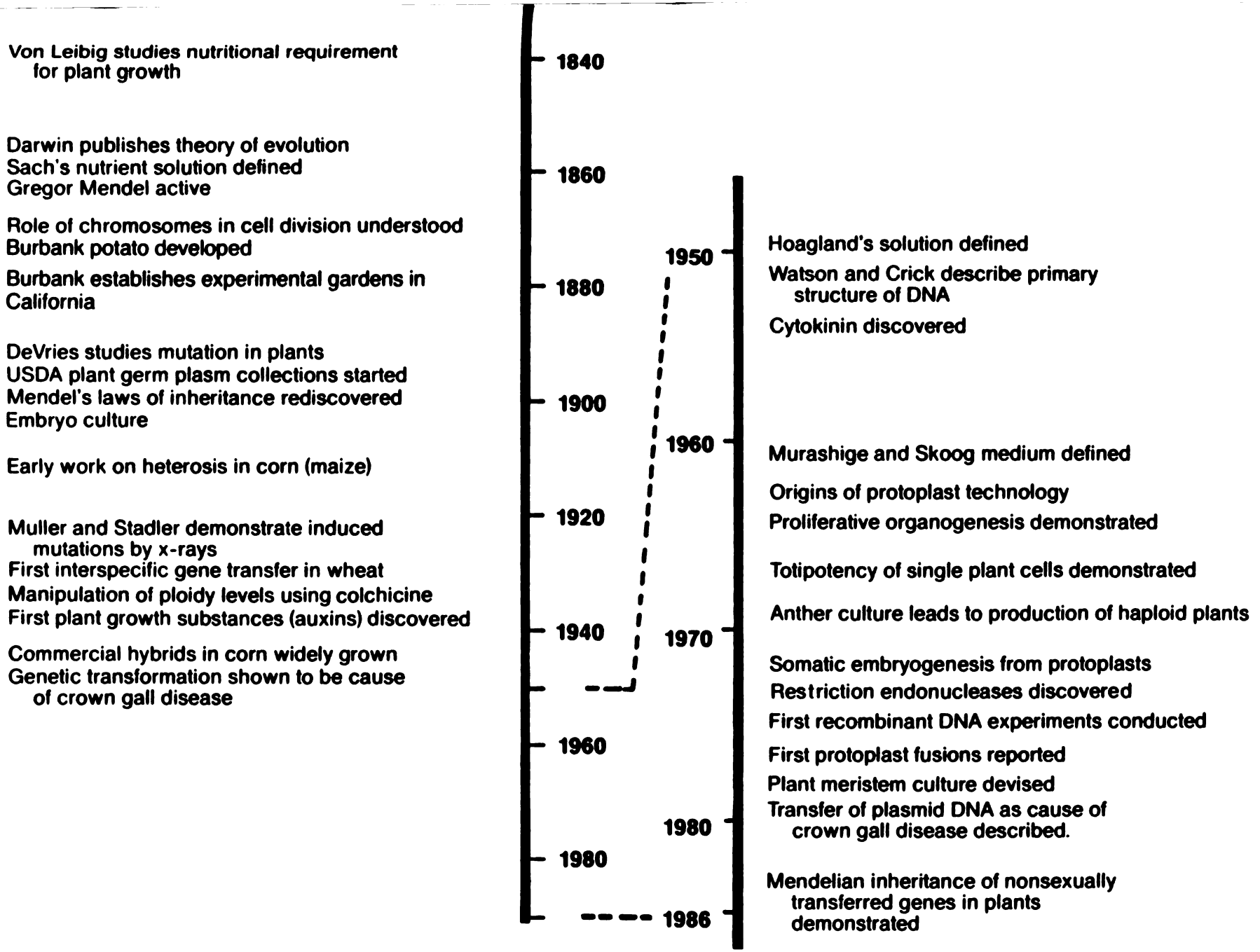
After a hybrid plant has been successfully recovered, differences in the number or compatibility of parental chromosomes may cause sterility. Cytogenetic manipulations have been instrumental in obtaining stable gene transfers. Sterility may result from incomplete or unstable pairing of chromosomes during cell division. It is sometimes possible to facilitate the cross by doubling the chromosome number of one or both of the parents, usually with the use of the mitotic inhibitor colchicine. Advanced generations are then back-crossed to the cultivated parent and monitored cytogenetically to select progeny with chromosomes from the donor species. Further manipulation may result in stable lines with a chromosome pair from the noncrop parent, either added to or substituted for a pair of the crop's chromosomes [(12) and Fig. 2].

For a desired gene from the donor to be incorporated into a chromosome of the crop variety, recombination must take place. If the two species are closely related, natural pairing and recombination may occur. Treatments such as irradiation can be used to induce translocation of a chromosome fragment from the donor to a crop chromosome, thus stabilizing the desired gene carried on the donor fragment (13).

Development of useful crop varieties. Even when successful, interspecific and intergeneric gene transfers make by sexual methods are laborious and time-consuming; moreover, other problems must be solved before a genotype useful for crop production is obtained. Even after six backcross generations in an intraspecific gene transfer, the natural process of recombination frequently will not separate tightly linked genes (14). This means that undesired traits that affect crop quality, yield, or adaptation may be carried along with the desired gene. The difficulty of separating linked deleterious genes has commonly limited the commercial potential of hybridization-derived varieties (15). Even if a desired gene is successfully separated from linked deleterious genes, its inheritance and expression may be unpredictably altered in the new genetic background.

Gene Transfer by Nonsexual Methods

Development of nonsexual methods for gene transfer in plants has been possible because plan cells, organs, and tissues


Von Leibig studies nutritional requirement for plant growth
1840
Darwin publishes theory of evolution
Sach's nutrient solution defined
Gregor Mendel active
1860
Role of chromosomes in cell division understood
Burbank potato developed
Burbank establishes experimental gardens in California
1880
DeVries studies mutation in plants
USDA plant germ plasm collections started
Mendel's laws of inheritance rediscovered
Embryo culture
1900
Early work on heterosis in corn (maize)
1920
Muller and Stadler demonstrate induced mutations by x-rays
First interspecific gene transfer in wheat
Manipulation of ploidy levels using colchicine
First plant growth substances (auxins) discovered
1940
Commercial hybrids in corn widely grown
Genetic transformation shown to be cause of crown gall disease
1960
1980
1986
1950
Hoagland's solution defined
Watson and Crick describe primary structure of DNA
Cytokinin discovered
1960
Murashige and Skoog medium defined
Origins of protoplast technology
Proliferative organogenesis demonstrated
Totipotency of single plant cells demonstrated
1970
Anther culture leads to production of haploid plants
Somatic embryogenesis from protoplasts
Restriction endonucleases discovered
First recombinant DNA experiments conducted
First protoplast fusions reported
Plant meristem culture devised
Transfer of plasmid DNA as cause of crown gall disease described.
1980
Mendelian inheritance of nonsexually transferred genes in plants demonstrated

can be cultured in vitro. Embryo rescue, which has been central to the success of wide crosses made with sexual methods, is one of many examples of such manipulations. Many of the methods for nonsexual gene transfer depend on our ability to produce in certain plant species (through a process called regeneration) fully differentiated plants from nonsexual tissues or organs. The starting material for regeneration can be pieces of leaves or stems or even various undifferentiated clumps of cells in culture. In some species, regeneration is possible starting even with a single somatic cell.

Concerted work to develop and exploit nonsexual methods for gene transfer to crops is a relatively recent development. Methods based on cell fusion have been studied for about 20 years. Approaches that use recombinant DNA, from which some agriculturally useful crop varieties may soon be obtained, became possible in 1983. There are few examples in which traits of any conceivable use in agriculture have been successfully transferred by nonsexual methods to crops. There are no samples of which we are aware in which crop varieties so derived are being used in commercial agriculture. The first field tests of crop plants modified by gene transfer with recombinant DNA were conducted in 1986. Thus, the introduction of these methods to crop improvement is in its infancy. Nevertheless, some of these methods have the potential to influence profoundly the future of crop improvement.

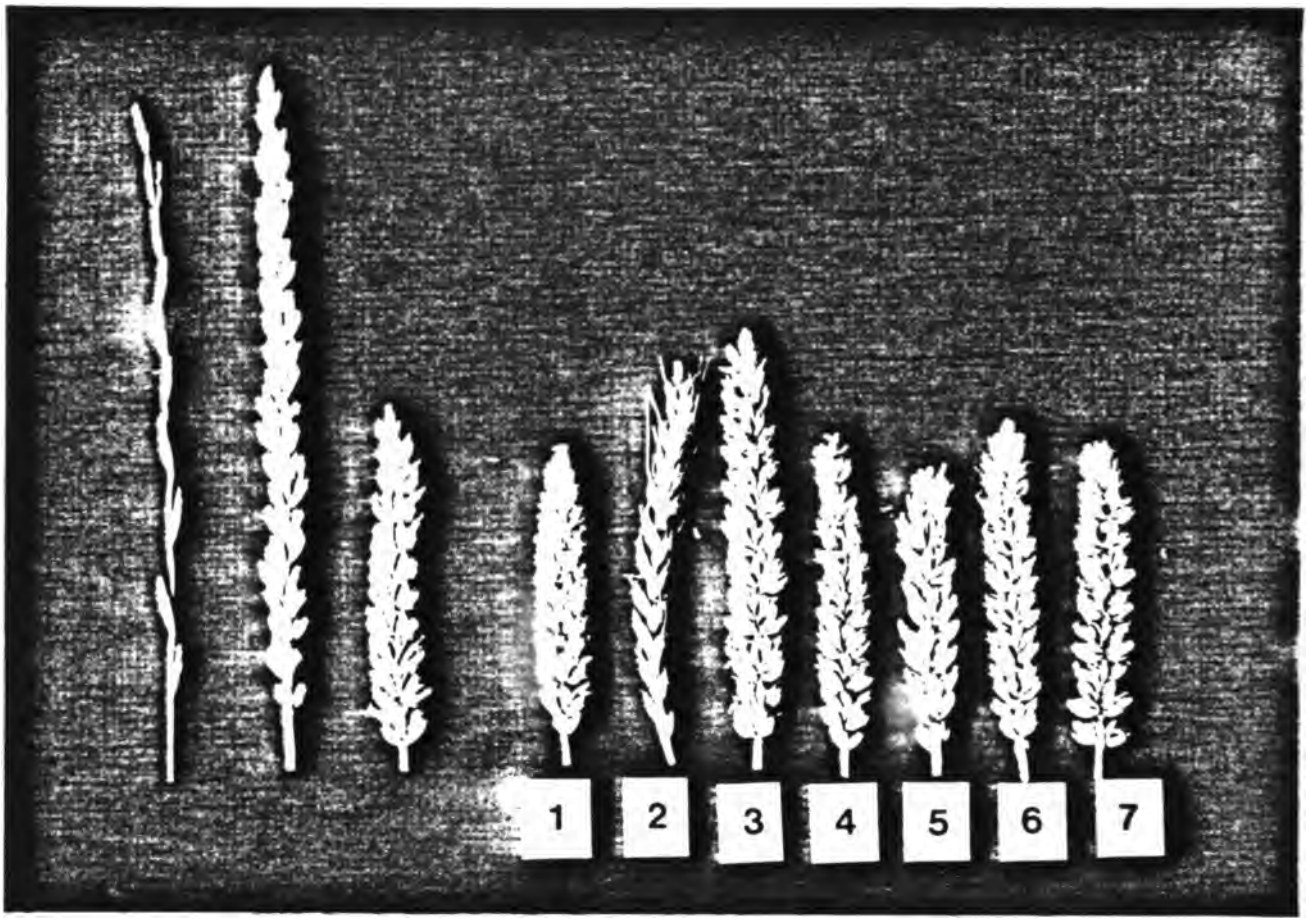
Cell fusion. In the 1960s, methods were developed that allowed preparation of large numbers of single plant cells stripped of their cell walls (protoplasts). Protoplasts from different species can be induced to fuse by exposure to certain chemicals or electric current. The resulting somatic hybrid may be grown in vitro to produce callus tissue from which in certain species a whole plant can be regenerated. The objective may be to combine the chromosomes of species that are sexually incompatible or, as a shortcut, to combine the nuclear genome of one species with the cytoplasm (that is, the organellar genomes) of another. Much work has been devoted over the past two decades to attempts to exploit cell fusion as a method to effect gene transfers between different species, but many problems have arisen and little commercial use in agriculture has resulted (16).

The greatest potential for the use of cell fusion methods may be in creating new crop varieties containing the nuclear genome of one species in the cytoplasmic background of another (nuclear transfer) or in a mixed cytoplasm with organelles from both species (cybrids). The plant mitochondrion and chloroplast each possess DNA, which encodes some of the proteins that make up the structure and metabolic machinery of the organelle. (The

majority of proteins found in organelles are imported, however, and are encoded by genes in the nucleus.) Although complex and not completely understood, certain agriculturally important traits are the result of interaction between the nuclear and cytoplasmic genomes. For example, a form of male sterility that is useful in commercial production of hybrid seeds results from nuclear-mitochondrial interactions. In species where cytoplasmic male sterility is not naturally found, making artificial combinations between nuclear and cytoplasmic genomes can result in such sterility. Well-studied cases include species of tobacco (**Nicotiana** spp.) and combinations of rapeseed (**B. napus**) nuclear genomes with cytoplasms from radish (**Raphanus sativus**) (17).

Much has been written about the potential for recombining genetic traits by cell fusion methods, and considerable work has been done to develop systems that will allow exploitation of these methods. It seems clear as a result of this activity, however, that many difficult questions remain to be answered. As with sexual hybridization, for example, incompatibility between parental species may result in hybrid instability (18). As explained above, there are several levels at which incompatibility can operate; somatic cell fusion bypasses some but not all the possible problems. Fusion may also severely compromise the ability of the resulting cell to undergo regeneration. The difficulties unique to cell fusion methods themselves notwithstanding, these methods share with plant breeding the drawbacks of imprecision. Deleterious genes will be transferred, and may be linked to, those encoding desirable traits. In cases where the target is a specific trait encoded by the nuclear genome, selection for the desired trait is made more difficult because a fusion-derived hybrid may have a mixture of cytoplasmic genes in addition to nuclear genes. Gene transfer methods based on cell fusion, like sexual hybridization, result in transfer of many genes. From the resulting hybrids, backcrossing or other schemes that use sexual methods are still needed to obtain plants with the desired combination of parental traits.

Gene transfer by manipulating DNA directly. Methods for tranferring DNA directly from one organism to another originated in experiments of the 1940s that established DNA as the chemical basis of genetic inheritance. Transfer of genes or chromosome segments in bacteria by sex factors (plasmids), viruses (transducing phage), or uptake of purified DNA (transformation) were well understood before in vitro gene splicing by recombinant DNA was demonstrated in 1973. Virally mediated gene transfer, direct DNA uptake, and microinjection have been successfully applied to animal cells. All of these approaches are also being applied to plants, but the approach that is most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advanced is a bacterially mediated DNA transfer system unique to higher plants.

Nonsexual DNA transfer techniques make possible manipulations that are outside the repertoire of breeding or cell fusion techniques. Genes can be accessed from exotic sources--plant, animal, bacterial, even viral--and introduced into a crop. Because the DNA elements that control gene expression can, and often must, be modified for proper function in the new host, it is possible to control timing, tissue specificity, and expression level of transferred genes. Endogenous plant genes may even be reprogrammed through the reintroduction of an engineered gene. Thus, nonsexual DNA transfer methods expand the sources of variability available for crop improvement to include all living things, and also allow manipulation to achieve quantitative control over gene expression. With methods available for chemically synthesized DNA or causing specific mutations in naturally occurring genes, entirely novel genes can be used. All of these effects can in principle be achieved with great precision.

Agrobacterium-mediated gene transfer. **Agrobacterium**-mediated gene transfer exploits the natural ability of **Agrobacterium tumefaciens** to transfer DNA into plant chromosomes (19). **A. tumefaciens** is a plant pathogen that transfers a set of genes encoded in a region called T-DNA of the Ti plasmid into plant cells at wound sites. The pathogen has a wide range of hosts among higher plants, including many dicotyledonous (broad-leaved) crop plants. The result of gene transfer is generally a tumorous growth called a crown gall in which the T-DNA is stably integrated into a host chromosome. The site of integration appears to be random. The tumor phenotype, which can be maintained indefinitely in tissue culture, results from the expression of genes on the T-DNA that alters the normal balance of growth substances (phytohormones) in transformed cells. The ability to cause crown gall disease can be removed by deletion of genes in the T-DNA without loss of DNA transfer and integration functions; and **Agrobacterium** strain that does not cause disease is said to be disarmed. In a disarmed strain, the DNA to be transferred is attached to border sequences that define the end points of an integrated T-DNA. To be active in T-DNA transfer, the **Agrobacterium** strain must also express a complex set of virulence genes also encoded on the Ti plasmid.

In the laboratory, disarmed **Agrobacterium** strains can be used to transfer genes to protoplasts with partially regenerated cell walls, suspension cell cultures, leaf pieces, and stem segments. The critical step is recovering a whole plant from cells that have acquired integrated T-DNA. Selectable markers

are used to identify and favor the growth of transformed cells. For example, the gene to be transferred is linked within the T-DNA to a gene conferring resistance to an antibiotic, such as kanamycin, which prevents plant growth. Plant cells that survive and can divide and undergo development in the presence of kanamycin are generally only those containing the engineered T-DNA. Because all the genes between the T-DNA borders are transferred, cells expressing the kanamycin-resistance gene are expected to contain any other genes engineered into the T-DNA region (19).

Gene transfer by means of engineered **Agrobacterium** strains has become routine in many laboratories for plants in the nightshade family such as tobacco, tomato, and petunia (19). Extension to other, more important crops has been difficult but progress is being made, particularly with species in other dicotyledonous families. In some cases, (for example, soybean) gene transfer has been demonstrated in cultured cells (20), but the ability to regenerate a complete plant from cultured cells containing T-DNA has not yet been reported. Although data have been cited that **Agrobacterium** can transfer T-DNA to monocotyledonous hosts (21), clear evidence of T-DNA integration exists only for asparagus, and, even in that case, no transformed plants have been described. Because A. **tumefaciens** does not induce crown galls on monocotyledonous plants, such as rice, corn, and wheat, other methods of gene transfer are being developed for these important crops.

Applications of Agrobacterium-**mediated gene transfer to agriculture.** In the 4 years since this method has been available, exciting progress has been made in applying it to the transfer of agriculturally useful genes. These include genes for insect and disease resistance and tolerance to safer herbicides. This is the only method of nonsexual gene transfer for which there are now practical and useful examples that are being tested and that are serious candidates for use in agriculture.

An early goal in the use of recombinant DNA for crop improvement has been to engineer bacterial or plant genes encoding enzymes that make crop plants tolerant to broad-spectrum, environmentally safer herbicides. One successful strategy has been to transfer a gene for an enzyme that complements the plant enzyme whose action is blocked by the herbicide. This has been done by engineering a bacterial gene so that its enzyme product is insensitive to the herbicide and then transferring it to the plant (22). Alternatively, the plant gene itself can be engineered so that the plant produces a larger amount of its own enzyme, making plants that can survive in the presence of the herbicide (23). Another strategy is to

engineer plants to express genes for enzymes that chemically detoxify the herbicide.

Bacterial genes for insecticidal proteins obtained from **Bacillus thuringiensis** have also been engineered into plants with exciting results. When certain insects feed on these plants, the bacterial toxin within the plant tissues kills them (24). The insecticidal protein is considered very safe; it has been widely tested and has been in use for a number of years in a crude powder form made from **B. thuringiensis** cultures. The toxicity of the protein is very specific. It is not toxic to mammals, plants, or even many kinds of insects.

Perhaps the most intriguing and unexpected result of agricultural interest has come from engineering plants with the gene encoding the coat protein from tobacco mosaic virus. Expression of this gene in tobacco and tomato plants resulted in resistance to infection by the virus (25). The mechanism of resistance is not understood, but, if it is a general result, we will have at hand a novel genetic approach to the control of virus diseases in plants. The use of insecticides for control of insects that spread certain plant viruses may also be avoided. To date, the search for chemical treatments to prevent of cure infections by plant viruses has been unsuccessful.

A key observation in these early tests of nonsexual gene transfer mediate by **Agrobacterium** is that genes transferred are inherited predictably. Also, the recipient plant varieties are apparently unchanged except for the acquisition of new traits encoded by the engineered genes. Thus it appears likely that the engineering itself will not compromise plant perfomance. This does not mean that undesirable results cannot occur. Occasional insertion of engineered DNA into genes that are required for proper functioning of the plant must occur at some frequency. Indeed, such insertions, if not lethal, could be an important scientific tool. The strong selection pressures applied in the gene transfer and regeneration processes, however, undoubtedly favor the recovery of normal plants. In summary, experience suggests that, when a desired trait has been introduced through **Agrobacterium** vectors, the resulting engineered plants are predictable, genetically stable, and useful.

Direct DNA transfer. Purified DNA can be used directly for plant transformation either by direct DNA uptake or by microinjection. Direct DNA uptake involves physicochemical reactions that result in DNA transfer to protoplasts. Microinjection is the mechanical introduction of DNA into cellular compartments with microscopic pipettes. Unlike methods based on **Agrobacterium** systems, direct gene transfer methods are

not subject to host range restrictions, but practically are limited by the need to recover a whole plant from the target cells or tissue.

Plant protoplasts can take up nucleic acids directly from the culture medium, a phenomenon first demonstrated with viral RNAs. Integration into plan chromosomes of foreign DNA introduced by direct uptake is a relatively rare event. Treatments, such as polyethylene glycol (PEG) and the application of electrical pulses (electroporation), which increase the permeability of membranes, can result in transformation frequencies of one transformant per thousand protoplasts (26). The combination of several uptake- enhancing treatments has increased transformation frequencies into the range of one in a hundred in at least one case (27).

With transformation frequencies near 1%, direct DNA uptake becomes an attractive method for gene transfer. In particular, plant species that either are not susceptible to **Agrobacterium** infection or are inefficiently transformed by it might be good candidates for direct DNA uptake if they can be regenerated from protoplasts. Although direct DNA uptake in other plant cells and tissues has been attempted, it has so far been successful only with protoplasts. Thus, application of direct DNA uptake to the cereals may be limited because regeneration of whole plants from protoplasts has not yet been achieved for many cereal species. However, there has been a recent report of regeneration of plants from rice protoplasts and a separate report showing expression of a foreign gene delivered by eletroporation to rice protoplasts (28). There are reports of stable transformation of maize cell lines by direct DNA uptake (26). Thus, prospects for methods to produce engineered plants by direct DNA uptake in the cereal crops are more encouraging.

Microinjection. Microinjection, the most recent addition to the repertoire of plant transformation methods, involves the introduction of DNA solutions under pressure into plant protoplasts by means of micropipettes. The key to successful transformation has been the development of methods for the immobilization of cells during injection and methods for their subsequent culture (29). In one study (30), cell lines cultured from microinjected tobacco protoplasts were shown to have integrated the foreign DNA sequences into the nuclear DNA; the average transformation frequency depended on whether the injections were intranuclear (14%) or cytoplasmic (6%). In another study (31), transformed cell lines cultured from intranuclear injections of alfalfa protoplasts were identified by screening for enzyme activity encoded by the foreign DNA; transformation frequencies ranged from 15 to 26% depending on the DNA injected. To date, there have been no reports to our

knowledge of transformed plants regenerated from cell lines obtained by protoplast microinjection.

Transformation of plant cells by microinjection has only been demonstrated with protoplasts. However, because microinjection is a physical means of introducing DNA, it should be capable of delivering genes into targets other than protoplasts. In this regard, it is important that intact cells have been shown to survive microinjection (32). As with direct DNA uptake, microinjection can in principle be used with any crop species from which whole plants can be obtained from single transformed cells.

Virally mediated gene transfer. Viral-based gene expression systems for animals have been developed, both for experimental and therapeutic uses. Some parallel effort has been made to develop vectors based on plant viruses for gene transfer into plants (33). In plants, viral-based vectors are not likely to stably transform plant cells because integration of viral genes into plant chromosomes has not been detected. The concept is that the engineered viruses would spread throughout a plant while expressing genes that confer some new trait. Results with the double-stranded DNA of cauliflower mosaic virus in which the strict requirements for gene expression and viral particle packaging have been taken into account in the design of the vector and a recent result from the use of Agrobacterium to deliver an infectious maize virus to plants show that virally mediated gene transfer in plants is possible (34). The contribution virally mediated gene transfer in plants will make to agriculture, however, is far from clear.

Summary and Conclusions

We believe that outside the agricultural research community few people appreciate how powerful a tool for crop improvement conventional plant breeding has been. The past use of gene transfers between species and even between genera is less appreciated. The history of scientific crop improvement shows how important technological innovation in the past has been in the enhancement of agricultural productivity. Future advances in crop improvement and the solution of the many problems facing agriculture today will depend on the wise use of all resources, including new technology, to advance fundamental knowledge about plants and apply this knowledge in the field.

The advent of recombinant DNA technology has focused scientific and public policy attention on gene transfers between species (35). As we have described, however, in plants, and particularly in crop improvement over the past century, interspecific and even intergeneric gene transfer is not new. Gene transfer by recombinant DNA is just the latest in a long history of increasingly more powerful methods available for crop improvement (Fig. 1).

Crop improvement has been a cornerstone of advances in agriculture and in the economic strength of the United States. Technological innovation and continued advances in fundamental science have driven efforts toward crop improvement. A return to prosperity in agriculture and a continuation of the primary role of U.S. agriculture in the economic strength of the nation depend on the availability and wise use of new technology. Research funding and other public policy decisions will profoundly affect the way new technology will be used, in both the public and private sectors of the agricultural research community, for crop improvement.

The research and public policy agenda. The new genetic engineering technologies have the potential to add precisely characterized genes to the preexisting germplasm with which a breeder has to work for a specific crop. Although the result may have considerable economic impact, each addition requires an extensive effort and must be considered as incremental to the 100,000 or so genes it takes to run a crop plant. Many desirable traits or phenotypes are conferred by the coordinated expression of a number of genes. To alter a plant trait in a desirable way, the correct genes to be manipulated must be chosen. This choice depends on an understanding of the biochemical bases of the process underlying the trait. Our knowledge of the biochemistry of plant traits important in agriculture is, generally speaking, very weak and a major area for future research.

Mechanisms for research funding are needed that bring the practice of scientific disciplines together and that focus on the advances in fundamental knowledge needed to ensure continuing advances in technology (36). Much of the attention within the agricultural research community in recent years has been given to the competing demands of plant breeding and molecular biology for scarce resources.

There are already numerous examples of ways in which recombinant DNA research has depended on past achievements in plant breeding and genetics. Proven superior crop varieties developed by plant breeders provide the genetic background for introduction of new genes developed in the laboratory. Isogenic lines (that is, plants differing in a single trait) are being used by molecular biologists to isolate genes related to specific traits such as disease resistance. Mobile genetic elements, first studied by genetic and cytogenetic analysis in the 1940s, have been used to isolate a specific gene from genetic variants in maize (37).

There is great potential for new methods to enhance the plant breeding process. Many genetic loci can now only be evaluated in a mature plant. If a transformation event can be identified in which a trait detectable in the embryo has become tightly linked to other traits, the size of experimental plots might be reduced by allowing selection at the embryo stage, or a breeding program may be accelerated by not having to complete a growing season to evaluate results. Either cloning a gene from a crop plant or inserting some unique piece of DNA can establish a specific chromosome position marker that along with restriction fragment length polymorphisms will lead to superior genetic maps for major crops (38). These maps will not only serve the breeder as a source of new markers to evaluate crosses but may also permit isolation of potentially important genes, which the plant geneticist can link to known markers.

Improved genetic maps together with traditional cytogenetic techniques may allow for gene isolation from microdissected chromosomes. Such research may lead to new technology for isolating poorly characterized genes, such as those involved in disease resistance, and may expand our knowledge or chromosome architecture and its effects on gene expression. Technological innovations motivated by the use of recombinant DNA in gene transfer may likewise advance cytogenetics. Specific chromosome transfers via microinjection might allow the transfer of complex multigenic traits. Organelle or nuclear transplants could allow traits, such as male sterilities, to be transferred or created. Such transfers, if successful, could be viewed as a more efficient way of making wide crosses between species. As a result, the range of species from which such transfers can be made will be extended.

Research funding should also address the major environmental issues that face agriculture. In particular, attention should be given to the ways in which the uses of genetic manipulation may reduce dependence on hazardous chemicals in agricultural production systems and promote long-term sustainability of highly productive agriculture (39). The possibilities are well illustrated by the recent reports of successful expression of genes conferring tolerance to safer herbicides, resistance to virus infection, and toxicity to insects.

Concerns over the regulation of the uses of recombinant DNA technology have been widely discussed. The unusual power of the technology, uncertainty over the behaviour to be expected from organisms modified in novel ways, and the past 40 years of experience with chemicals in the environment make it reasonable and indeed desirable that genetically modified organisms be introduced cautiously. The regulatory framework should, however, be based on a thorough understanding of the scientific issues and should be properly calibrated to the likely risks and rewards (40). As we have shown here, there is a wealth of past experience with interspecific gene transfer in crop plants that is relevant to this question and is in general reassuring.

Finally, consideration of the technology used in gene transfer highlights the critical importance of collecting, preserving, and characterizing the world's germplasm for plants and microorganisms. Many of the advances that will enhance agriculture in the future will probably be made as the result of entirely unforeseeable ideas and manipulations by future generations of scientists. We must preserve the raw material from which our successors will work.

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