Prospects for Human Gene Therapy

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Summary. Procedures have now been developed for inserting functional genes into the bone marrow of mice. The most effective delivery system at present uses retroviral-based vectors to transfer a gene into murine bone marrow cells in culture. The genetically altered bone marrow is then implanted into recipient animals. These somatic cell gene therapy techniques are becoming increasingly efficient. Their future application in humans should result in at least partial correction of a number of genetic disorders. However, the safety of the procedures must still be established by further animal studies before human clinical trials would be ethical.

Gene therapy, the insertion into an organism of a normal gene which then corrects a genetic defect, has been carried out in fruit flies (*1*) (*Drosophila melanogaster*) and mice (*2*). How soon gene therapy might be available for the treatment of human genetic diseases and what criteria should be used in determining when clinical trials should begin are issues examined in this article. Several investigators are now preparing protocols for clinical trials of gene therapy in seriously ill patients (*3*). Since most of these protocols will be based on the use of retroviral vectors as a delivery system, these structures will be emphasized. It may well be, however, that one of the other delivery systems described below, or a new one not yet developed, will be the procedure of choice in the future.

Gene Therapy in Lower Species

The most elegant system thus far demonstrating successful gene therapy is the work in *Drosophila* (*1*). The transposable genetic element, the P factor, has been used to transfer a normal gene coding for the enzyme that produces the wild-type red eye color in *Drosophila* embryos which have a genetically defective gene. The result is that the treated flies acquire normal eye color. Similar transfer experiments under way use other genes. Despite considerable searching, transposon-like elements have not been identified in vertebrates. Retroviruses, however, are structurally and functionally similar in many ways to the mobile genetic elements found in lower organisms, and retroviral vectors have now been used to transfer functioning genes into mouse bone marrow cells.

The first genetic "cure" reported in a mammal (*2*) was in a strain of mice, called *little*. These have a mutation that results in reduced serum levels of growth hormone, and the mice are therefore dwarfs. The equivalent human disease is pituitary dwarfism. Hammer *et al.* (*2*) succeeded in inserting a rat growth hormone gene into the cells of these mice in such a way that the gene is expressed at a high level. The deficiency in growth hormone was corrected, and the animals grew rapidly, but the gene was not controlled appropriately, and gigantism resulted—namely, a mouse one-and-a-half times as large as a normal animal. A major research effort is focusing on how to correctly regulate transferred genes.

Gene Therapy in Humans

Human disease candidates for gene therapy. Pituitary dwarfism in humans is not a reasonable initial candidate. Genes making hormones that circulate in the bloodstream are probably not appropriate for early attempts at gene therapy in humans. First, the normal feedback controls in DNA that regulate the expression of hormone genes in the body are not now known. Therefore, physiologically correct levels of hormone production would probably not be possible. Second, it would be easier and safer to use recombinant DNA manufacturing techniques to produce sufficiently large quantities of hormone so that the active polypeptide itself could be given to the patient. Hormone levels could then be titrated precisely.

At first, clinical investigators thought that the human genetic diseases most likely to be the initial ones successfully treated by gene therapy would be the hemoglobin abnormalities (specifically, β-thalassemia) because these disorders are the most obvious ones carried by blood cells, and bone marrow is the easiest tissue to manipulate in vitro (*4*). Regulation of globin synthesis, however, is unusually complicated. Not only are the embryonic, fetal, and adult globin chains carefully regulated during development, but also the α- and β-globin-like chains are always maintained in a 1 to 1 ratio despite the fact that the α- and β-globin loci are on different chromosomes. To understand the regulatory signals that control such a complicated system and to develop means for obtaining controlled expression of an exogenous β-globin gene will take considerably more research effort. The recent development of a mouse model for β-thalassemia should aid these investigations (*5*).

Gene therapy should be beneficial primarily for the replacement of a defective or missing enzyme or protein that must function inside the cell that makes it, or of a deficient circulating protein whose level does not need to be exactly regulated (for example, factor VIII). Early attempts at gene therapy will almost certainly be done with genes for enzymes that have a simple "always-on" type of regulation. Three genes are the initial prime candidates: hypoxanthine-guanine phosphoribosyl transferase (HPRT), the absence of which results in Lesch-Nyhan disease; purine nucleoside phosphorylase (PNP), the absence of which results in a severe immunodeficiency disease; and adenosine deaminase (ADA), the absence of which results in severe combined immunodeficiency disease. For all three, the clinical syndrome is profoundly debilitating. The defect in each is found in the patient's bone marrow (although the severe central nervous system manifestations of Lesch-Nyhan disease are due to absence of HPRT in brain cells and probably cannot be corrected with current techniques). In all three there is no, or minimal, detectable enzyme in marrow cells from patients homozygous (or hemizygous) for the defect, and the production of a small fraction of the normal enzyme level should

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be beneficial. Furthermore, a mild overproduction of enzyme should not be harmful to the cell. In addition, in all three the gene has been cloned and a complementary DNA is available.

Since combined immunodeficiency due to a defect in the ADA gene in T lymphocytes can be corrected by infusion of normal bone marrow cells from a histocompatible donor, selective replication of the normal T cells appears to take place (*6*). This observation offers hope that defective bone marrow can be removed from a patient, the normal ADA gene inserted into a number of cells through gene therapy, and the treated marrow reimplanted into the patient where it may have a selective growth advantage. There is also evidence that marrow cells containing HPRT ($HPRT^+$) may have a selective advantage (in both mice and humans) over cells that do not ($HPRT^-$) (*7*). If selective growth occurs, no ablation of the patient's own marrow would be necessary. If, however, corrected stem cells have no growth advantage over endogenous ones, then partial or complete marrow destruction (either by irradiation or by other means) may be required in order to allow the corrected marrow cells an environment favorable for expansion.

Ethics. The ethics of gene therapy in humans has been discussed for many years (*8*) and is being widely debated at present (*9*). Essentially all observers have stated that they believe that it would be ethical to insert genetic material into a human being for the sole purpose of medically correcting a severe genetic defect in that patient—that is, somatic cell gene therapy. Attempts to correct germ cells (that is, to permit the new gene to be passed on to the patient's children) or to enhance or improve a "normal" person by gene manipulation do not have societal acceptance at this time (*9*). However, somatic cell gene therapy for a patient suffering a serious genetic disorder would be ethically acceptable if carried out under the same strict criteria that cover other new and experimental medical procedures (*10*). The techniques that are now being developed for human application are for somatic cell, not germ line, gene therapy.

The question examined here is: What criteria should be used in evaluating gene therapy protocols? Three general requirements, first presented in 1980 (*10*), are that it should be shown in animal studies that (i) the new gene can be put into the correct target cells and will remain there long enough to be effective; (ii) the new gene will be expressed in the cell at an appropriate level; and (iii) the new gene will not harm the cell or, by extension, the animal. These three requisites, summarized as delivery, expression, and safety, will each be examined in turn.

Delivery

At present, the only human tissues that can be used for gene transfer are bone marrow and skin cells. No other cells can be extracted from the body, grown in culture to allow manipulation, and then successfully reimplanted into the patient from whom the tissue was taken. In the future, as more is learned on how to package the injected DNA and to make it tissue- or even cell type–specific, the intravenous route would be the simplest and most desirable. Attempting to give a foreign gene by injection directly into the bloodstream is not advisable with our present state of knowledge, since the procedure would be enormously inefficient and there would be little control over the DNA's fate (*11*).

Studies are considerably more advanced with bone marrow than skin cells as a recipient tissue for gene transfer. Bone marrow consists of a heterogeneous population of cells, most of which are committed to differentiation into erythrocytes, lymphocytes, megakaryocytes, and so on. Only a small proportion (0.1 to 0.5 percent) of nucleated bone marrow cells are stem cells (that is, cells that have not yet differentiated into specific cell types and which divide as needed to maintain the marrow population). In gene therapy, stem cells would be the primary target. Because they are low in number and are not recognizable, a delivery system for transferring a gene into stem cells must be efficient.

Techniques for transferring cloned genes into cells can be grouped in four categories: (i) viral, both RNA viruses (or retroviruses) and DNA viruses (for example, SV40, adenovirus, and bovine papilloma); (ii) chemical, such as calcium phosphate–mediated DNA uptake; (iii) fusion, that is, fusion of DNA-loaded membranous vesicles, such as liposomes, red blood cell ghosts, or protoplasts, to cells; and (iv) physical, that is, microinjection or electroporation. Each technique is valuable for certain types of experiments, but none can yet be used to insert a gene into a specific chromosomal site in a target cell. Fusion techniques are the least well characterized and will not be discussed. As noted, retroviral-based vectors appear to be the most promising approach at present for use in humans.

Viral Techniques

RNA viruses (retroviruses). There are a number of advantages of vectors derived from retroviruses as a gene delivery system. First, up to 100 percent of cells can be infected and can express the integrated viral (and exogenous) genes; this is in contrast to chemical methods where, although most cells take in the DNA, as shown by positive assays after 48 hours, only one cell in 10^3 to 10^7 stably expresses the exogenous gene. Second, as many cells as desired can be infected simultaneously; 10^6 to 10^7 is a convenient number for a simple protocol. Third, under appropriate conditions the DNA can integrate as a single copy at a single, albeit random, site, whereas the chemical and physical techniques often result in the insertion of multiple copies of the transferred gene, all linked head-to-tail in tandem repeats. Fourth, although integration is random with respect to the host genome, it is precise with respect to the viral genome—that is, the structure of the integrated DNA is known. Fifth, the infection and long-term harboring of the retroviral vector usually does not harm cells. Finally, a wide and controllable host range is available. A number of retroviral vector systems have been developed. Here we concentrate on vectors based on Maloney murine leukemia virus (MoMLV).

1) Life cycle and structure. The details of the life cycle of retroviruses have been reviewed recently (*12*). In brief, the retrovirus, composed of an RNA-protein core and a glycoprotein envelope, enters a cell where the RNA acts as a template for the reverse transcription of the genetic information into a double strand of DNA. This DNA can precisely integrate as a single copy, called a provirus, at a random location in the genome of the host.

Although much has been learned about the regulatory features of retroviruses, uncertainties remain. Those features of the proviral structure that are thought to be necessary for transcription and transmission of the viral genome are (see Fig. 1): a long terminal repeat (LTR) sequence on each end, containing regulatory signals for initiating and terminating transcription, sequences required for reverse transcription and others for proviral integration; short sequences (called here, for short, r^- and r^+) immediately adjacent to each LTR and necessary for reverse transcription; the packaging sequence called ψ in MoMLV, necessary for the viral RNA to be packaged into an infectious viral particle; and the donor (D) and acceptor (A) splice sites.

Retroviral RNA is synthesized from the proviral DNA by the host cell's own RNA polymerase. A portion of this RNA is used in the cell's translational machinery to synthesize the viral proteins that go into the final viral particles along with the genomic RNA. These viral particles bud off from the cell and can then infect other cells.

From experimental studies as well as the existence of a number of naturally occurring defective viruses, it is known that almost all of the regions coding for viral proteins (*gag*, *pol*, and *env* in Fig. 1) can be deleted and some or all of these sequences replaced with other DNA. Once the viral genes are deleted, the retroviral vector becomes defective. In order to obtain infectious viral particles, a cell harboring a defective provirus must be infected with a "helper" virus, which carries all the viral functions needed—that is, the genes for *gag*, *pol*, and *env*.

2) Use as gene delivery system. The proviral DNA for the desired retrovirus [commonly either MoMLV or murine sarcoma virus (MSV)] is isolated and inserted into a convenient plasmid. The viral genes can then be replaced with the exogenous genes of choice by standard recombinant DNA techniques. This construct is used to transfect tissue culture cells (for example, NIH 3T3 cells) by a convenient gene transfer procedure (for example, calcium phosphate). After infecting the cells with a helper virus (such as intact MoMLV), infectious viral particles, possessing both the retroviral vector and the helper virus, bud off from the cells into the surrounding medium. This particle-containing supernatant is collected and used to infect bone marrow cells in culture or, more simply, freshly extracted bone marrow is incubated directly with the cells budding the viral particles. The marrow cells are removed and injected intravenously into a mouse whose bone marrow has been killed by x-rays (lethally irradiated). The animal is then studied to determine if the transferred marrow cells express the desired gene from the vector.

3) Successful gene transfer into adult mice. Joyner *et al.* (*13*) have successfully used this procedure to transfer a functional gene for neomycin resistance (*neo*^{r}) into mouse hematopoietic progenitor cells by use of a MoMLV retroviral vector. The presence and expression of this gene in granulocytic progenitor cells rendered these cells resistant to the neomycin-like antibiotic G418 as determined by in vitro colony assays. Treated cells were injected into lethally irradiated mice; Southern blot analysis and colony assays showed that the *neo*^{r} gene is present and functional in the spleens of the recipient animals (*13*).

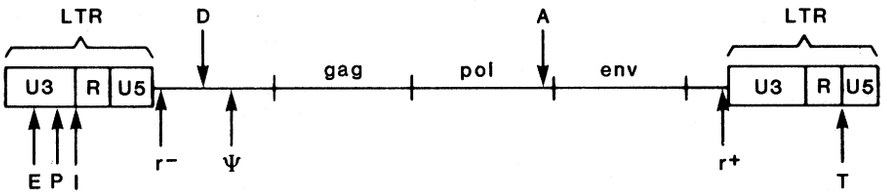

Fig. 1. Simplified structure of MoMLV retroviral provirus DNA. Abbreviations: E, enhancer; P, promoter; I, initiation (Cap) site for viral RNA synthesis; r$^-$, replication initiation site for minus DNA strand (transfer RNA binding site); D, donor splice site; ψ, packaging sequence; A, the major acceptor splice site; r$^+$, replication initiation site for plus DNA strand (purine-rich site); T, terminal [poly(A) addition] site for viral RNA synthesis; LTR, long terminal repeat; U3, R, and U5 are portions of the LTR; *gag*, group-specific (that is, viral core) antigens: p15, p12, p30, and p10; *pol*, RNA-dependent DNA polymerase (reverse transcriptase); and *env*, envelope proteins: gp70, p15E, and R. (Not drawn to scale)

An improvement on this procedure would be to treat bone marrow cells with a retroviral particle that could deliver the vector but which would not itself produce a spreading infection. Mann *et al.* (*14*) have developed a technique for accomplishing this goal. The regulatory signal ψ (Fig. 1) contains a sequence, the exact size and structure of which are not yet known (*15*), that must be present in the viral RNA for it to be packaged into a viral particle. A helper virus was constructed with this sequence deleted (ψ$^-$) by making use of convenient restriction endonuclease sites (Bal I and Pst I) flanking the ψ sequence in MoMLV. The ψ$^-$ helper is able to produce all the viral proteins required to make a particle, but the particle does not package its own RNA. Since the retroviral vector has a ψ sequence, it is packaged. Consequently, the particle can just infect once; it is only a delivery system for the vector, not an infectious agent.

In order to use the ψ$^-$ helper virus conveniently, a line of NIH 3T3 cells was established with the helper proviral DNA permanently integrated (*14*); ψ$^-$ helper viral RNA is produced constitutively. The transfection of this cell line (called ψ-2) with the retroviral vector DNA results 48 hours later in a supernatant that contains viral particles with only the vector.

Williams *et al.* (*16*) have used the ψ-2 cell line to place a functioning *neo*^{r} gene into the hematopoietic cells of adult mice. Freshly extracted murine bone marrow was layered onto ψ-2 cells producing a retroviral vector called MSV DHFR-NEO, which contains genes for dihydrofolate reductase (DHFR) and *neo*^{r} in an MSV backbone. The marrow and ψ-2 cells were incubated for 48 hours under standard incubation conditions; similar results were obtained when bone marrow cells with the ψ-2 MSV DHFR-NEO cell layer were incubated for 6 days under Dexter-type conditions (*17*). The viral particles that budded off into the supernatant contained the MSV DHFR-NEO vector but, to the extent that could be determined, no ψ$^-$ helper viral RNA. Ten days after lethally irradiated mice were injected with the treated bone marrow cells, analysis of the regenerating spleens showed that the mice carried the MSV DHFR-NEO proviral DNA in their hematopoietic cells. Individual spleen colonies, each arising from a single stem cell, were generated by injecting an estimated one to ten stem cells into another group of lethally irradiated mice. Cells from individual colonies were able to produce spleen colonies in a secondary group of lethally irradiated animals. These mice also were shown to carry MSV DHFR-NEO DNA in their total spleen DNA and, in each case, to have the same integration site restriction pattern as the colony from the primary mouse. These data show that the delivery system is effective, at least for mouse bone marrow cells. Preliminary evidence indicates that the *neo*^{r} gene is expressed (*16*).

Southern blot analysis of total spleen DNA with a number of restriction enzymes revealed in some cases a small number of proviral integration sites. This result suggests that only a few infected stem cells were proliferating to repopulate the irradiated spleen. Secondary transfers of individual colonies showed that only 7 of 48 colonies (15 percent) contained the *neo*^{r} gene. This is a lower limit since an occasional colony might have been formed from endogenous stem cells that survived the irradiation. These data suggest that the present bone marrow procedure might still be made more efficient as a delivery system.

A similar retroviral vector system based primarily on MoMLV has been developed by Verma and his co-workers (*18*). In their ψ$^-$ helper virus they substituted an amphotrophic (that is, wide host

range) *env* gene for the MoMLV *env* gene, which produces a particle coat with a narrow host range. This helper viral construct is called pSAM. Miller *et al.* (*19*) built a retroviral vector containing a full-length complementary DNA for the human enzyme HPRT. This vector, called pLPL, was cotransfected along with the ψ^- helper, pSAM, into HPRT$^-$ BALB/3T3 cells. One clone (c7cl) was obtained that produced high levels of viral particles containing the HPRT vector. Injection of these cells into lethally irradiated mice resulted in animals that continued to produce HPRT-vector particles for at least 6 months (*19*). The infectious particles resulted from the presence of low levels (<0.1 percent of the HPRT-vector virus) of packageable helper virus along with the injection of MoMLV as additional helper which led to multiple rounds of replication in the host. In addition, human HPRT enzyme was detected in spleen cells.

4) Shortcomings of retroviral delivery systems. The evidence indicates that retroviruses can be used as a reasonably efficient delivery system. A gene therapy procedure, however, also requires a reliable system. In most of the work reported to date, a number of cells are found to contain altered proviral DNA. The biggest problem appears to be that retroviruses have a strong propensity for deleting sequences during virus replication (*19a*). Many vectors have been ineffective because the foreign DNA is partially or totally removed from the construct or is rearranged. For example, Joyner and Bernstein (*20*) have used the Friend spleen focus-forming virus as a potential vector system for hematopoietic cells. Constructs containing a thymidine kinase (TK) gene in the *gag* region and an intact *env* gene (gp55) were used, along with MoMLV as helper, to obtain viral particles. The particles were injected into lethally irradiated mice and also layered onto rat TK$^-$ (LTA) cells. Southern blot analysis of the integrated proviral DNA in erythroleukemic spleens demonstrated vector constructs with intact gp55 genes but deleted TK sequences, whereas TK$^+$ LTA clones possessed intact TK genes but deleted or rearranged gp55 sequences. In other words, in no case could a provirus be found that still contained both the TK and gp55 genes. Even the successful MSV DHFR-NEO vector, which produces *neo*^{r} expression in mice, has lost a portion of its DHFR gene during production of the viral particles (*16*). Several approaches are being tried to circumvent this problem of instability (*19a*).

5) Properties still needed for an optimal delivery system. An ideal delivery system not only would be stable but also would be tissue-specific. When a genetic disorder is in the hematopoietic system, then the isolated bone marrow can be treated. But no other tissue, except skin cells, can be removed, treated, and replaced at present. Since many viruses are known to infect only specific tissues (that is, to bind to receptors that are present only on certain cell types), a retroviral particle containing a coat glycoprotein that recognizes only human hematopoietic stem cells would permit the retroviral vector to be given intravenously with little danger that cells other than those in the marrow would be infected. Such specificity could permit the liver and brain, for example, to be treated individually. In addition, the danger of inadvertently infecting germ cells could be eliminated. One problem, however, is that cell replication appears to be necessary for integration. It would not be possible to infect nondividing brain cells, for example, as far as we now know.

The optimal system not only would deliver the vector specifically into the cell type of choice but would also direct the vector to a predetermined chromosomal site. Specific insertion into a selected site of a chromosome by means of homologous recombination can be readily achieved in lower organisms but appears to be a formidable task in mammals, whether retroviral vectors or plasmid-based vectors are used. Present evidence suggests that homologous site-specific integration occurs at a very low level, when it occurs at all, in mammals (*21*).

DNA viruses. Viruses, such as SV40, with DNA as the nucleic acid in their core have been employed for several years as gene transfer vectors (*22*). A conditionally nonreplicating adenoviral vector has recently been developed that will efficiently infect animal and human cells (including hematopoietic cells) with the result that one or a few copies of the recombinant virus are integrated into the host cell's genome (*23*). Whether adenoviral vectors will be as efficient as retroviral vectors, or will offer other advantages as a gene transfer delivery system, remains to be determined. One subcategory of DNA viruses should be mentioned: bovine papilloma virus (BPV) (*24*). This viral DNA replicates extrachromosomally so that BPV-based vectors may prove to be useful for maintaining genes in cells in a nonintegrated manner. Transfection of hematopoietic cells with BPV-vectors has not yet been reported.

Chemical Techniques

The other procedure under active consideration for insertion of genes into human bone marrow cells is calcium phosphate–mediated DNA uptake. The original procedure of Graham and van der Eb (*25*) was modified by Wigler *et al.* (*26*) in order to insert into the genome of mammalian cells growing in culture a fragment of DNA carrying one or more genes. A number of genes have been used including the herpes simplex TK gene complementing TK$^-$ cells, the DHFR gene protecting against the drug methotrexate, and the *neo*^{r} gene protecting against the antibiotic G418.

Procedure. Transfection is carried out by pipetting a suspension of DNA, complexed into small precipitates with calcium phosphate, onto a monolayer of cells growing in a tissue culture dish (*26*). A number of techniques are used to increase the efficiency of transfection in different cell types: for example, diethylaminoethyl dextran can be employed instead of calcium phosphate or the cells can be shocked with glycerol after 2 hours of incubation (*27*). The efficiency of the process varies with the cell line. Under optimal conditions and very receptive cells (for example, mouse L cells), one cell in 10^2 to 10^3 can be obtained that has integrated and expressed the exogenous DNA. Because the usual efficiency is 10^{-5} to 10^{-7}, a procedure is required to detect the occasional transfected cell. In other words, a gene must be present that can protect the cell from a lethal selective agent that is added to the incubation medium or that complements a genetic defect (HPRT or TK, for example). The transfected cell will survive while all others are killed. Attempts to obtain transfected cells without selective pressure have generally been unsuccessful.

Transfection appears to work poorly in suspension cells, namely bone marrow cells. Efficiencies can only be estimated, but the value is probably one cell in 10^6 or 10^7. Using the powerful selection system offered by the mutant DHFR gene (isolated from 3T6-R400 cells) that provides exceptional resistance to methotrexate, Carr *et al.* (*28*) reported that the calcium phosphate transfer technique can be successfully employed to obtain mouse bone marrow cells that contain a functional exogenous DHFR gene. The permanently transfected cells can partially repopulate a lethally irradiated mouse. These results support the studies of Cline *et al.* (*29*) who reported successful transfer of a functional DHFR gene into the bone marrow of mice. However,

the presence of the DHFR gene has not been confirmed with DNA hybridization studies and, until such experiments are reported, the efficiency of the calcium phosphate procedure is uncertain.

Shortcomings of chemical techniques. If a chemical technique for gene transfer were used in a protocol designed for humans, the predicted results appear discouraging. Recovery from bone marrow of approximately 10^{10} nucleated cells (of which 10^7 to 10^8 are stem cells) can routinely be obtained from patients for marrow transplantation. Efficiency of 1 in 10^6 would mean that only 10 to 100 stem cells would be transfected. Reinsertion of these cells into the total stem cell pool of 10^8 to 10^9 cells would be very unlikely to have any noticeable effect on a patient's course unless there was an extraordinary selective advantage for the treated cells. Any human gene therapy protocol that uses chemical means for transfection would have to establish, therefore, that either a few transfected stem cells might have a detectable beneficial effect on the patient's course or that the investigator has improved substantially the efficiency of the procedure for human bone marrow cells.

Physical Techniques

Microinjection (*30*) and electroporation (*31*) are the two principal classes of physical techniques. Electroporation, a relatively new technique, is the transport of DNA directly across a cell membrane by means of an electric current. It has been used to transfer a variety of genes into a number of different cells including the immunoglobulin κ gene into B cells (*31*). Its potential for human gene therapy is uncertain.

Microinjection has been used for a number of years and has the advantage of high efficiency (up to one cell in five injected can be permanently transfected). However, the distinct disadvantage is that only one cell at a time can be injected. Transfection of a large number of hematopoietic stem cells is not feasible. Even if a stem cell could be recognized it would have to be fixed to a slide for injecting. The effect of attaching, injecting, and subsequent detaching is unknown. Microinjection of mouse erythroleukemia (MEL) cells is difficult, although possible (*32*), and these cells are much easier to manipulate in culture than are bone marrow cells.

Transfer of genes into mouse eggs. An area where microinjection has had spectacular success is in transferring genes into fertilized mouse eggs (*33*). Gordon *et al.* (*34*) first demonstrated that if plasmid DNA is microinjected into one of the two pronuclei of a recently fertilized mouse egg, and the ovum is then placed into the oviduct of a pseudopregnant female, the egg could develop into a normal mouse carrying the plasmid DNA in every cell of its body. Furthermore, the injected DNA can be transmitted to offspring in a normal Mendelian manner. Mice carrying an exogenous gene in their genome are called "transgenic."

Hammer *et al.* (*2*) used this technique to partially correct a mouse with a defect in its growth hormone production. By attaching a rat growth hormone gene to an active regulatory sequence (specifically, the promoter that normally directs the synthesis of metallothionein messenger RNA in mice), they obtained a recombinant DNA construct that actively produces growth hormone in the genetically defective mouse. Although the level of growth hormone production is inappropriately controlled—that is, influenced by signals that normally regulate metallothionein synthesis—these experiments do show that microinjection can be used as a delivery system that can put a gene into every cell of an animal's body.

Nonapplicability for humans. Should the technique of microinjecting a fertilized egg be employed for human gene therapy at the present time? The answer is no on three grounds: the procedure has a high failure rate, can produce a deleterious result, and would have limited usefulness. Microinjection has a high failure rate because the majority of eggs are damaged by the microinjection and transfer procedures so that they do not develop into live offspring. In one recent experiment involving microinjection of an immunoglobulin gene (*35*), 300 eggs were injected, 192 (64 percent) were judged sufficiently healthy to be transferred to surrogate mothers, only 11 (3.7 percent) proceeded to live birth and 6 (2 percent) carried the gene. These results are from a highly experienced laboratory in which thousands of identical eggs from the same hybrid cross of inbred mice have been injected over a number of years. The mice were chosen precisely because they gave the best results for gene transfer by microinjection. Experience with attempts to microinject growth hormone genes into livestock eggs have met with a number of major biological and technical problems (*36*). Successful gene transfer by microinjection of human eggs, without a long period of trial and error experimentation, is extremely unlikely.

Second, microinjection of eggs can produce deleterious results because there is no control over where the injected DNA will integrate in the genome. Lacy *et al.* (*37*) showed that the integration of an exogenous rabbit β-globin gene in transgenic mice could sometimes occur into a chromosomal location that results in expression of the β-globin gene in inappropriate tissue, namely, muscle or testes. There have been a number of cases reported where integration of microinjected DNA has resulted in a pathological condition (*38*). Although there is no control over where exogenous DNA will integrate in any gene transfer procedure, the damaging effect caused by a harmful insertion site could be great when it occurs in the egg but may be negligible when it occurs in one or a few of a large number of bone marrow cells.

Third is limited usefulness. Not only is it of questionable ethics to experiment on human eggs because of the expected losses, but even if "success" were obtained, it would be applicable primarily when both parents are homozygous for the defect. When the parents are both carriers, only one fertilized egg out of four would result in an affected child (*39*). Since a homozygous defect cannot yet be recognized in an ovum, and since the procedure itself carries such a high risk, it would be improper to attempt any manipulation in this situation. Furthermore, most of the very serious genetic disorders result in infertility (or death before reproductive age) in homozygous patients. Consequently, there would be little use for the procedure even if it were available. A different approach for human gene therapy is required.

Expression

The second criterion for evaluating a human gene therapy protocol is that there be appropriate expression of the new gene in the target cells. Even when a delivery system can transport an exogenous gene into the DNA of the correct cells of an organism, it has been a major problem to get the integrated DNA to function. A vast array of cloned genes have been introduced into a wide range of cells by the several gene transfer techniques discussed above. "Normal" expression of exogenous genes is the exception rather than the rule.

Active exogenous promoters in transgenic mice. Microinjection of fertilized eggs with exogenous DNA to obtain transgenic mice carrying an expressing gene has resulted in several spectacular successes, but also in a considerable number of unpublished failures. Thus far

only four genomic promoters have been reported to show significant activity: metallothionein (*2*), transferrin (*33*), immunoglobulin (*35*), and elastase (*40*). However, essentially any complementary DNA can be attached to an active promoter, such as metallothionein, and the coding sequence will usually be expressed in a transgenic mouse under the control of that promoter.

Why are most promoters inactive after microinjection into mouse oocytes? At least one promoter has been examined in this regard: mouse β^{maj} globin. The sequences are found to be heavily methylated in mouse tissues where they are inactive but relatively unmethylated in tissue culture cells where they are active (*41*). Therefore, the mouse zygote appears to respond to this foreign DNA by covering it with methyl groups which remain on the DNA throughout the lifetime of the animal. Attempts to decrease the methylation of the genomic DNA by treating adult mice carrying an exogenous β-globin promoter with the hypomethylating drug 5-azacytidine have been essentially unsuccessful (*41*). The metallothionein promoter, however, even if methylated, can remain active (*42*). Why some promoters are inactivated by methylation, or other mechanisms, while others are not is not known.

Expression from retroviral vectors. If a retroviral vector is used for gene transfer, the transcriptional signals in the retrovirus's own LTR's can be used (Fig. 1). Expression of exogenous genes carried by retroviral vectors into bone marrow cells has been reported by three laboratories. The two studies in which a *neo*^{r} gene was expressed in mouse bone marrow were described above (*13, 16*). The most extensive data, however, are from Willis *et al.* (*43*). A homozygous Lesch-Nyhan (LN) lymphoblast cell line was used to determine whether an HPRT$^-$ human hematopoietic cell could be corrected by a retroviral vector containing a functional HPRT gene. The LN cells have all the characteristics of a cell line totally defective in HPRT, specifically a disruption in their inosinate cycle that leads to a high purine production and a number of other metabolic abnormalties (*44*). LN cells infected with viral particles containing the HPRT vector could be rescued in selective medium. Seventeen HPRT$^+$ clones were isolated and studied. These cell lines had HPRT levels ranging from 4 to 23 percent of the normal level, and the abnormalities associated with a deranged inosinate cycle were partially to nearly completely corrected (*43*). In a corollary study, viral particles containing the HPRT-vector were used to infect mouse bone marrow cells that were then injected into lethally irradiated mice (*19*). Both human HPRT proteins and chronic production of HPRT-vector particles were detected in the hematopoietic tissue of the mice.

A problem must still be overcome, however. Even though expression of HPRT and *neo*^{r} genes has been obtained in the hematopoietic tissue of irradiated mice, the efficiency of the combined delivery-expression system is poor. If 15 percent of stem cells can be infected and if 4 to 23 percent of normal expression can be obtained in them, can sufficient enzyme be synthesized to be of benefit to a patient? The issue, once again, is whether or not the treated cells will have a selective growth advantage in the patient's body. If they do not, then, either the patient's own bone marrow must be partially or totally eliminated before reimplantation of the treated cells or the gene therapy protocol must demonstrate at least some expression in nonirradiated animals. It must be recognized, however, that, in the absence of a true animal model for a given genetic disease, it might be difficult or impossible to demonstrate selective growth advantage except in human patients.

Use of enhancers to increase expression. How can the level of expression be increased and properly regulated? One key element may be the enhancers. These are DNA sequences usually 50 to 150 base pairs in length that increase the expression of the adjacent gene 10 to 1000 times (*45*). A retrovirus has its own enhancer immediately upstream from its promoter in the LTR (Fig. 1). Enhancers are known to be species-specific (*46*). A primate enhancer (for example, the 72 base pair repeat from SV40) is several times more active in primate tissue culture cells than in rodent cells. Likewise, a mouse enhancer (for example, the 73 base pair repeat from MSV) is more active in rodent cells than in primate cells. The promoter acted upon does not influence the species specificity (a mouse β-globin promoter and a primate SV40 promoter are both activated more by a primate enhancer in primate cells than in rodent cells), although different promoters can be enhanced to different extents (*47*). Retroviral vectors designed for therapeutic application in humans may need primate, or even human, enhancing sequences rather than the mouse ones that are now used.

Some enhancers may even be tissue-specific (*48*). With a tissue-specific enhancer it may not be necessary to develop a cell-specific delivery system. The DNA could be integrated into all cells but only be expressed significantly in that tissue in which the enhancer is active. Even more precision may be achieved if one could place a tissue-specific coat on a retroviral particle that would direct the virus into the target cell, along with a tissue-specific (and possibly even a developmental-time-period-specific) enhancer in the construct itself.

Systems like globin undoubtedly have other regulatory regions in addition to enhancers which recognize cellular factors that are involved in control. Much information still needs to be learned about the regulatory signals in these multigene families.

Expression from plasmid-based expression vectors. If a chemical gene transfer technique is used as a delivery system, then the gene must be inserted into an appropriate expression vector. An expression vector is a plasmid (usually pBR322) in which the complementary DNA (or genomic gene) of interest is inserted together with regulatory signals. A typical expression vector would be composed of a promoter (for example, from the mouse metallothionein gene), the complementary DNA of choice, a splice site and polyadenylation site (necessary for correct processing of the transcribed RNA), and an enhancer.

Plasmid-based expression vectors containing an enhancer have not yet been used to transfect bone marrow cells. Therefore, how effective expression might be is unknown. The inefficiency of the presently available delivery systems for these vectors was discussed above.

One additional complication is that calcium phosphate–directed transfection, as well as microinjection, does not usually result in the integration of a single copy of the expression vector. The plasmid DNA vector appears to be ligated or replicated, or both, inside the cell to form a long head-to-tail structure called a tandem repeat (*49*). This tandem repeat, which can be a few or up to hundreds of copies in length, is randomly inserted usually in one site in the genome. The tandem repeats may produce problems for genes requiring intricate regulation because of the uncertainty as to how many of the copies are active.

Regulation by genomic control signals. Can either plasmid-based expression vectors or retroviral vectors be used to transfer genes that are controlled by the gene's own genomic regulatory sequences? Plasmid-based expression vectors in transgenic mice do respond to normal physiological control signals in some cases. Metallothionein-promoted genes express primarily in the liver, the

normal tissue for metallothionein synthesis, and can be induced by cadmium, as occurs in vivo for the endogenous gene; however, they do not respond to steroids, which are another physiologic inducer in vivo (*50*). An immunoglobulin gene is expressed in the spleen, the correct in vivo tissue, and not in liver (*35*). A mouse-human β-globin fusion gene expresses in hematopoietic tissue (*51*).

In tissue culture cells, a number of plasmid-based expression vectors have demonstrated at least a degree of normal regulation. For example, the human β-globin gene with approximately 1 kilobase of genomic 5′ flanking sequence can be induced (along with endogenous mouse globin) in a transfected MEL cell (*52*). The level of expression is not as high as that of the normal endogenous β-globin gene, suggesting that other regulatory signals are needed. However, transfection of MEL cells with cosmids carrying 30 to 40 kilobases of human genomic DNA containing the human β-globin gene does not result in higher expression of human β-globin messenger RNA (*53*).

Miller *et al.* (*54*) obtained encouraging results when they placed a rat growth hormone complementary DNA together with 237 bases of genomic 5′ flanking sequence into the *env* region of the HPRT-vector already described above. This growth hormone gene was regulated in rescued HPRT$^-$ fibroblast cells by its own genomic promoter and regulatory sequences as shown by (i) stimulation by glucocorticoid and thyroid hormones, which are normal in vivo regulators, and (ii) equal activity whether the fragment was placed in the same direction or opposite to the vector's LTR's (*54*). Expression of the vector in an animal has not yet been studied.

These data provide hope that vectors can be built with all the genomic regulatory signals necessary to produce correctly controlled expression in target cells. In the future, one might use only selected portions of a retrovirus in order to construct a delivery and integration system that would place one copy of the vector DNA into the target cell's genome. Expression would be controlled by the exogenous gene's own genomic regulatory signals. One possible problem is size: it appears that MoMLV constructs must not be over 9 to 12 kilobases in order to be packaged. Since 2 or 3 kilobases are necessary for essential function, only 6 to 9 kilobases are available for insert. This amount may be adequate, but further studies are needed to determine the answer (*55*).

Importance of chromosomal location. A major question that remains is: How important is chromosome location? Integration of a proviral structure can in some cases activate a downstream gene, as can occur with oncogenes. This problem could be eliminated by deleting the enhancer and promoter regions from the 3′ (right-hand) LTR in the retroviral vector. One round of reverse transcription could then occur which would result in double-stranded retroviral DNA with both LTR's defective. The retroviral vector DNA would then integrate with no transcription initiation signals. Therefore, expression would have to be controlled by exogenous signals in the inserted gene, and no downstream activation of other genes could take place.

Certainly an integration site that disrupts an important gene or regulatory sequence would normally be detrimental. How often this would occur must be determined by experiment. It is probable though that in most such cases the insertional event would diminish the fitness of the recipient cell so that it would be outgrown by normal cells.

Are there only certain active chromatin regions that can allow expression of a gene? Or could an expression vector take its own "active domain" with it so that essentially any location would be acceptable? The answers to these questions are still not known.

Safety

The third and final criterion for evaluating a human gene therapy protocol is that the delivery-expression system be safe.

Retroviral vectors. Although retroviruses have many advantages for gene transfer, they also have disadvantages. One problem is that they can rearrange their own structure as well as exchange sequences with other retroviruses. In the future it might be possible to modify retroviral vectors in such a way that they become less unstable. At present, however, there is the possibility that a retroviral vector might recombine with an endogenous viral sequence (*56*) to produce packageable, infectious recombinant virus. Properties that such a recombinant would have are unknown, but the potential homology between retroviral vectors and as-yet unknown primate cancer retroviruses or human T-cell leukemia viruses might be sufficiently close so that possible recombinants should be sought. There is, however, a built-in safety feature with the mouse retroviral vectors now in use. These mouse structures have a very different sequence from known primate retroviruses, and there appears to be little or no homology between the two (*57*). Therefore, a potentially "safe" proviral vector construct might be one composed of mouse LTR's, with their enhancer and promoter regions deleted, and a human gene controlled by the appropriate human genomic regulatory signals.

With the present constructs, three types of experiments ought to be carried out before any retrovirus-treated bone marrow is injected into a patient. These protocols, designed to test the safety of the delivery-expression system, are necessary since once treated bone marrow is reinserted into a patient, it and all retroviruses that it contains are irretrievable.

First, studies in vitro with human bone marrow are needed. Marrow cultures infected with the therapeutic vector should be tested for a period of time for the production of recombinant viruses. Any infectious virus isolated should be studied for possible pathogenicity.

Second, studies in vivo with mice are needed. Since many retroviral vectors are constructed from mouse retroviruses, and expression studied in mouse bone marrow transplanted into lethally irradiated (or nonirradiated) mice, these animals should be followed to determine if genomic rearrangement or the site of chromosomal integration has resulted in any pathologic manifestations or the production of any infectious viruses.

Third, studies in vivo with primates are needed. A protocol similar to the one planned for human application should be carried out in primates, not just mice, because the endogenous proviral sequences in primate, including human, DNA are different from those in mouse DNA. Therefore, the nature of any viral recombinants would be different. Treated bone marrow should be reimplanted into primates, the successful transfer of intact vector DNA into hematopoietic cells demonstrated, the expression of at least small amounts of gene product verified, and the existence of infectious recombinant viruses sought and, if found, analyzed.

Plasmid-based expression vectors. The calcium-phosphate procedure for transferring a plasmid-based expression vector into human bone marrow has not yet been demonstrated to be an effective delivery system. However, the procedure itself does not appear to represent a significant risk of harm. In theory, of course, a stem cell could be altered to make it carcinogenic so that it would still be necessary to follow treated mice over time to determine the likelihood of pathology. Primate studies, however, would appear not to be necessary.

Review Procedure

The initial clinical protocols designed to carry out gene therapy in patients will probably be evaluated in the following way. Under current Department of Health and Human Services regulations for the protection of human research subjects, a human gene therapy protocol must be reviewed by the Institutional Review Board at the investigator's home institution. In addition, because of the widespread public interest and concern in this area, the National Institutes of Health has announced (*58*) that any federally funded gene therapy experiment involving recombinant DNA must first be approved by the NIH after review by the Recombinant DNA Advisory Committee (RAC). Prior to review by RAC, proposals will be examined by a special RAC working group on human gene therapy (*59*). In addition, the Food and Drug Administration could regulate the DNA used in a human trial as a biological drug, analogous to polynucleotide interferon inducers, interferons, and vaccines (*60*). The Food and Drug Administration is currently exploring its regulatory responsibilities in this area (*61*).

Representative Albert Gore's proposal for a President's Commission on the Human Applications of Genetic Engineering (*62*) has just passed both houses of Congress in a modified form. This commission, if signed into law, would probably concern itself primarily with matters of policy and procedure rather than the review of individual recombinant DNA research proposals (*63*); the initial protocols, however, might be of particular interest to the commission in helping it to define the scope of its efforts.

Conclusion

It now appears that effective delivery-expression systems are becoming available that will allow reasonable attempts at human gene therapy. These systems are based on treatment of bone marrow cells with retroviral-vectors carrying a normal gene. The safety of the procedures is the remaining major issue. Patients severely debilitated by being homozygous for a defect in the gene for one of the enzymes HPRT, PNP, or ADA are the most likely first candidates for gene therapy.

It is unrealistic to expect a complete cure from the initial attempts at gene therapy. Many patients who suffer from severe genetic diseases, as well as their families, are eager to participate in early clinical trials even if the likelihood is low that the original experiments will alleviate symptoms. However, for the protection of the patients, particularly since those with the most severe diseases and, therefore, the most ethically justifiable first candidates, are children, gene therapy trials should not be attempted until there are good animal data to suggest that some amelioration of the biochemical defect is likely. Then it would be necessary to weigh the potential risks to the patient, including the possibility of producing a pathologic virus or a malignancy, against the anticipated benefits to be gained from the functional gene. This risk to benefit determination, a standard procedure for all clinical research protocols, would need to be carried out for each patient.

In summary, institutional review boards should carefully evaluate therapeutic protocols to ensure that the delivery system is effective, that sufficient expression can be obtained in bone marrow cultures and in laboratory animals to predict probable benefit, even if small, for the patient, and that safety protocols have demonstrated that the probability is low for the production of either a malignant cell or a harmful infectious retrovirus. Once these criteria are met, I believe that it would be unethical to delay human trials. Patients with serious genetic diseases have little other hope at present for alleviation of their medical problems. The issues of germ line therapy and enhancement engineering need to be debated widely in society, but arguments that genetic engineering might someday be misused do not justify the needless perpetuation of human suffering that would result from an unnecessary delay in the clinical application of this potentially powerful therapeutic procedure (*64*).

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63. J. Jensen, personal communication.
64. This position is, in fact, consistent with the existing regulation which states: "The IRB should not consider possible long-range effects of applying knowledge gained in the research . . . as among those research risks that fall within the purview of its responsibility" [45 *CFR* 46 (March 8, 1983) 46.111(a)(2)].

RESEARCH ARTICLE

Heterochronic Mutants of the Nematode *Caenorhabditis elegans*

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Significant evolutionary change in multicellular organisms may arise from mutations in genes that control temporal or spatial patterns of development. Much of the evolutionary variation in morphology and life history among related species has been proposed to result from "heterochrony," that is, from differences in the relative timing of developmental events (*1–3*). In some cases, radical morphological differences between species appear to result from simple differences in developmental timing. The isolation of mutations that lead to heterochrony may identify genes that control temporal patterns of development and also that could mutate to introduce heterochronic variation between species.

We have sought heterochronic mutants of the nematode *Caenorhabditis elegans*. The relatively simple anatomy and virtually invariant cell lineage of *C. elegans* (*4–6*) facilitate the detailed comparison of mutant and wild-type developmental patterns. Observation of living worms in the light microscope with the use of Nomarski differential interference contrast optics allows individual cells to be studied. Changes in temporal patterns of development can be characterized by examining the fates of individual cells at specific times during development. Each cell can be recognized by its lineage history and position, and its developmental fate can be defined by its morphology (an indicator of cell type), and in the case of blast cells, by the number and types of its progeny cells (*4*). The four larval stages of *C. elegans* are characterized by stage-specific patterns of cell division and differentiation (*4, 5*) and stage-specific cuticle formation (*7*).

Many mutants of *C. elegans* have been identified that are abnormal in cell lineage (*8*). The mutant *lin-4(e912)*, isolated because of its inability to lay eggs, displays multiple and complex cell lineage defects, with some patterns of cell division occurring repeatedly and others not occurring at all (*8, 9*). These mutant cell lineages, as well as other defects of *lin-4(e912)* can be interpreted as heterochronic; for example, the times of expression of certain cell division patterns are altered, while other cell division patterns occur at their normal times. Because a defect in egg-laying appeared to be one consequence of the heterochronic development of *lin-4(e912)*, we have screened other egg-laying defective mutants (*10*) for similar alterations in the stage specificity of developmental events. In this article, we describe the heterochronic developmental defects

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