Gene Therapy

Application of Molecular Biology

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Preface

Over the millennia of human cultural evolution, it has been gradually recognized that certain traits are inherited and that these may lead to human disease and suffering. However, the processes governing human heredity were not known until early in the twentieth century. Despite the revelations of Mendel, Watson and Crick, and more recently recombinant DNA, therapeutic applications were not forthcoming.

We are now on the threshold of a revolution; a revolution that will have major ramifications for human medicine. Giant strides in our understanding of genes and the elements that control their expression have made it possible to intervene directly in the genetic process.

The subject of this book is gene therapy with emphasis on application to human disease. With the exception of infectious diseases, genetic diseases have been perhaps among the best understood in biochemical terms, yet least accessible to therapeutic intervention. Advances in recombinant DNA technology and molecular and cellular biology have now made it feasible to correct genetic defects. The field of gene therapy is progressing rapidly and the present work provides a "snapshot" for those unfamiliar with the territory. Advanced undergraduates, graduate students, and physicians in training are among the audience to whom this book is directed.

Chapters 1 and 2 provide an introduction to the topic, background information on molecular and cellular biology, and an historical perspective. Chapter 3 delves into the genetic basis of inherited disorders and diagnosis of genetic diseases. Chapter 4 covers the technology and progress made in gene transfer in vitro, and Chapter 5 expands this topic to describe progress made on in vivo application in various mammalian species. Chapter 6 describes progress made with bone marrow transplantation, an important therapeutic intervention that may be required for some forms of gene therapy but curative in its own right in a number of disorders. Chapter 7 covers other tissues as potential targets for human gene transfer. Chapter 8 covers advances in the generation of transgenic animals. This technology will be used for the generation of animal models of human diseases. This has obvious application to correction of the defects by gene therapy. While it is unlikely that germline intervention will be attempted in humans in the near future, work with transgenic animals provides much important information on gene regulation and expression in vivo. There are also obvious veterinary applications of this work. Chapter 9 reviews a number of human genetic disorders and discusses the prospects for gene therapy. Despite the promise of gene therapy, it is important to place this technology into

perspective by comparison with alternative therapies. An overview of therapeutic alternatives is the subject of Chapter 10. Chapter 11 introduces social and ethical aspects of this technology. Finally, Chapter 12 briefly describes future directions of this exciting area of biomedical research. Key references are provided at the end of each section, and the reader is encouraged to consult these sources for the technical details and depth that cannot be presented in a survey of this type.

Gene therapy is a technology in its infancy. Despite the tremendous potential of the technology, it is unclear how widely it will be applied to the treatment of human disease. If techniques can be refined and therapy proven to be safe and effective, it is likely that no area of medicine will be untouched. The intention of this work is to provide background for the avalanche of discoveries that will deluge this field in the years to come.

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Contents

Introduction and Overview of Human Gene Therapy

1

Genetic diseases account for a significant amount of human suffering. About 4% of all infants born in the United States and Canada are affected by a birth defect. Approximately 1 in 200 births is affected by a chromosomal abnormality whereas 1 to 2 per 100 is affected by a single gene disorder, most often recessive in character. The remainder of the disorders are presumed to be multifactorial in etiology, that is, involvement of more than one gene and/or influenced by environmental factors.

The human genome is contained on 46 chromosomes of various sizes (Figure 1.1) containing over three billion base pairs comprising an estimated 50,000 to 200,000 genes. Although most genetic disorders are individually rare, several thousand such disorders are known. Over 3000 different human diseases are known or suspected to result from defects in single genes. Many such diseases are well known and rather prevalent; for example, sickle cell anemia, cystic fibrosis, Duchenne/Becker muscular dystrophy, thalassemia, and phenylketonuria. Others are less common but no less devastating to their victims; for example, the severe combined immunodeficiency (SCID) syndromes, Lesch-Nyhan syndrome (hypoxanthine phosphoribosyl transferase deficiency), and the various lipid and carbohydrate storage diseases.

Current therapy for genetic disorders as a group is woefully inadequate (Table 1.1, see Chapter 10 for a discussion of alternative therapies). Current therapy offers reasonable prospects for a normal and healthy life for only a handful of diseases. Present treatment of genetic diseases attempts to correct symptoms or secondary biochemical defects. For example, β-thalassemia syndromes result from a defect in β-globin gene production with severe anemia attributable to markedly ineffective erythropoiesis. Current therapy calls for blood transfusion—some 100 to 150 units are transfused from infancy through early childhood with the attendant risks of infection with hepatitis and the human immunodeficiency viruses. Many of these patients succumb to cardiomyopathy secondary to iron overload in young adulthood. Further complications of iron overload include liver failure and multiple endocrinopathies (eg, diabetes mellitus, delayed puberty). To combat these complications, chelation of excess iron is attempted by prolonged subcutaneous administration of desferroxamine. This is suboptimal therapy.

Patients with a severe combined immunodeficiency syndrome often die in infancy or early childhood from overwhelming infection. Symptomatic therapy exists in the form of antibiotics for particular infections and doses of immune globulin to shore up the failing immune system. Bone marrow transplantation

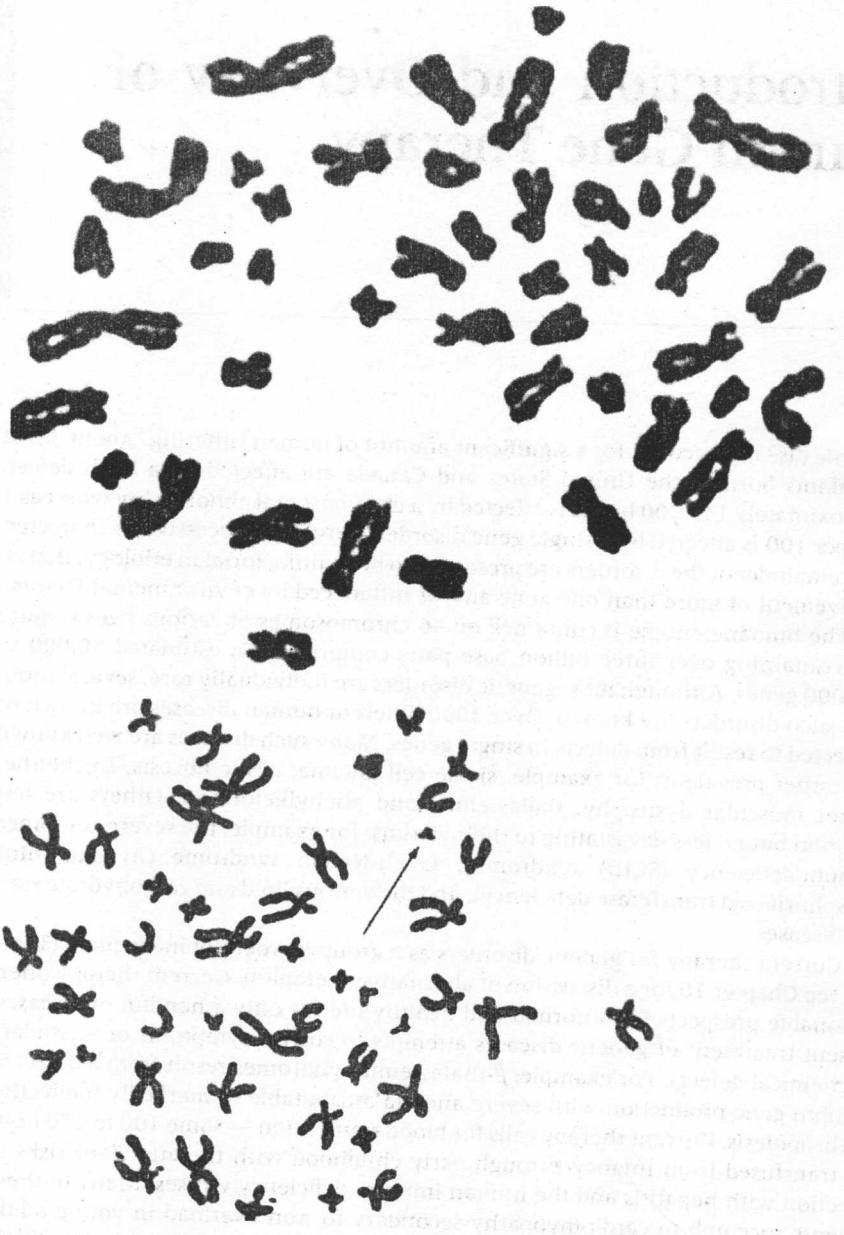

Figure 1.1. Metaphase spreads of human chromosomes. The top picture shows the first metaphase published in 1956 by Tjio and Levan in which the correct number of chromosomes in the human was established. The lower metaphase shows *(arrow)* a Philadelphia chromosome (an aberrant chromosome no. 22), to date the most characteristic and consistent karyotypic marker of human leukemia (chronic myelogenous leukemia). *Reprinted from Sandberg AA: The Chromosomes in Human Cancer and Leukemia. New York, Elsevier, 1990.*

Table 1.1. Examples of Nongenetic Therapies for Genetic Diseases[a]

Disease	Therapy
Sickle cell anemia	Blood product replacement
β-thalassemia	Bone marrow transplantation
Endocrine disorders Diabetes mellitus Thyroid hormone deficiency Growth hormone deficiency Congenital adrenal hyperplasia	Hormone replacement
Phenylketonuria, galactosemia	Diet
Severe combined immunodeficiency	Immunoglobulin replacement, antibiotics, isolation in sterile environment, bone marrow transplantation, enzyme replacement
Lysosomal storage diseases	Supportive care, bone marrow transplantation, enzyme replacement
Lesch-Nyhan syndrome (HGPRT deficiency)	Supportive care
Cystic fibrosis	Antibiotics, pancreatic enzyme replacement, aggressive pulmonary toilet

[a] For further discussion of this topic, see Chapter 10.

provides definitive therapy in the 20% to 30% of patients with a suitable bone marrow donor. More radical, and decidedly nonstandard, therapy completely isolates the patient from external pathogens in a sterile atmosphere; all substances with which the patient comes in contact, including food and clothing, must be sterilized. The patient may never feel the direct touch of another human being, including members of his or her own family, lest he/she be contaminated by the potentially lethal microorganisms harbored by all of us. This is suboptimal therapy.

Phenylketonuria is currently treated by adherence to a strict diet during infancy and childhood. This diet contains reduced amounts of phenylalanine. Because phenylalanine is an essential amino acid, it cannot be entirely eliminated from the diet. However, all proteins contain phenylalanine, making the diet difficult to follow. Patients able to adhere to the diet do not suffer the characteristic central nervous system abnormalities characterized by severe mental retardation and are able to lead reasonably normal and healthy lives. Studies suggest that those who adhere to the diet in adulthood have reduced ill effects. Pregnant women who do not follow the diet will have retarded offspring even if the babies themselves are not affected with the disease.

Diseases like cystic fibrosis and sickle cell anemia are treated symptomatically. Cystic fibrosis patients suffer pancreatic insufficiency and contract frequent pulmonary infections due to thick, tenacious secretions. They benefit from antibiotics, hydration, and vigorous pulmonary toilet. Sickle cell patients suffer from painful crises essentially due to poor circulation. Sickled blood cells containing polymerized hemoglobin are unable to adequately oxygenate tissue (Figure 1.2). Such patients also benefit from antibiotics (splenic infarction leads to an increased susceptibility to infection by certain (encapsulated microorganisms), hydration, and analgesia. Because narcotic analgesics are often needed in large doses, drug addiction is a not uncommon complication. This symptomatic therapy, too, is inadequate.

For patients with Lesch-Nyhan syndrome, a disorder of purine metabolism

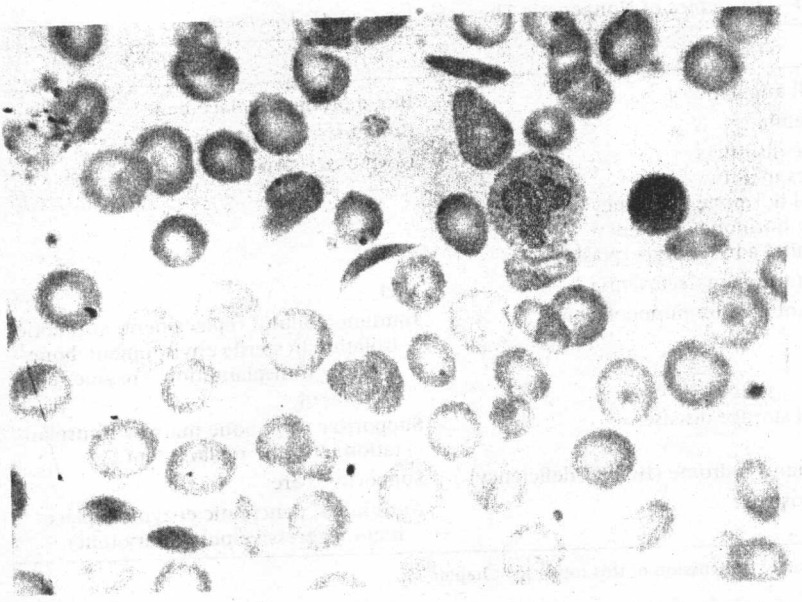

Figure 1.2. Scanning electron micrograph of sickled human erythrocytes. Normal cells are biconcave discs. Sickled cells are contorted by the presence of aggregations of hemoglobin fibers within the cell and often assume long crescent shapes. *(Courtesy of Dr. James Feeley, Washington Hospital, Fremont, CA.)*

characterized by self-mutilating behavior, mental retardation, choreoathetosis, and hyperuricemia, no therapy whatever exists, aside from using allopurinol to correct the hyperuricemia; this medication does nothing for the neurologic deficits. Similarly, there is no proven treatment for any of the wide variety of storage diseases with their accompanying mental retardation and multiorgan failure due to buildup of early or middle products of defective metabolic pathways. These few examples serve to illustrate the need for new genetic disease therapy.

Gene therapy encompasses a broad range of technologies that eventually may be applied to a diverse group of genetic diseases. Gene replacement therapy has theoretical appeal since it is directed at the mutant gene instead of symptoms or secondary events in the pathogenesis of disease. Recent advances in molecular biology, in particular, gene cloning and exogenous gene expression, have elevated the prospect of gene therapy from the realm of science fiction to the possible in the not-so-distant future.

Genetic diseases fall into a number of categories. Single genes may be inherited as autosomal recessive or as autosomal dominants or may be sex-linked, that is, inherited on the X (or Y) chromosome (see Chapter 3). Normal genes may be expressed in several ways. Patterns of gene expression vary from constitutive (always on) to simple "on-off" mechanisms, to complex tight regulation of two or more subunits of an enzyme. It might be anticipated that those diseases most amenable to early attempts at gene therapy would be those where a single gene product is missing (recessive inheritance) and where normal expression is constitutive or simple on-off.

Introduction of exogenous genes might correct a genetic defect (a) by random insertion in the genome or (b) by specifically targeting a gene using homologous

Table 1.2. Potential Forms of Gene Therapy

Random gene insertion or addition
Targeted approaches
Gene modification (in situ)
Gene substitution or replacement
Gene activation (modification of control elements in situ)
Combined approaches

recombination (Table 1.2). Terminology used to describe the genetic changes include:

1. Gene insertion, where a normal copy of the gene is introduced randomly (nonspecifically) into a target cell or tissue; in this case a normal gene is added and the abnormal (usually dysfunctional) gene remains.
2. Gene modification, where a gene is corrected in situ to its normal form.
3. Gene "surgery" or substitution, where a defective gene is excised and physically replaced with its normal counterpart.

Endogenous genes might be activated, in which case control elements of genes are modified to direct expression of an existent normal structural gene. In addition, a combination of one or more of the above processes might be used to open novel metabolic pathways or target the end organ(s) affected by the pathologic process of the primary genetic defect.

These types of genetic alterations may involve genetic material that codes directly for the defective gene product or that affects regulation of existing genes, for example, via suppression or enhancement of production of particular proteins. Gene modification might entail reverse mutagenesis via chemical or molecular mutagens and be directed at one or a few base pairs contributing to the genetic defect. Gene surgery could be envisioned as site-specific recombination which replaces a defective gene with a cloned natural gene.

There are four conceptual types of genetic manipulation in humans (Table 1.3):

1. Somatic gene therapy addresses correction of the disorder via manipulation of somatic (body) cells of the individual. In practice only some cells will be affected, in particular, those where the gene defect is predominantly manifest, eg, red cells for sickle cell anemia or liver cells for phenylketonuria.
2. Germline gene therapy results in correction of the genetic defect in the patient's reproductive tissue such that the disorder would no longer be heritable by his or her offspring. If performed at an early embryonic stage the patient, also, would have the defect corrected.
3. Enhancement gene engineering, where additional genetic material is in-

Table 1.3. Potential Types of Genetic Manipulation

Somatic cell gene therapy
Germline gene therapy
Gene enhancement therapy
Eugenic manipulation

serted into an individual with no known genetic ailment with the objective of enhancing a known genetic characteristic. A simple example is insertion of an additional gene for growth hormone to make a human being larger. A more justifiable application might be gene implantation to prevent occupational diseases in individuals at increased risk or to make normal cells more resistant to the toxic effect of chemotherapy.

4. Eugenic genetic engineering, with an attempt to "improve" human traits such as intelligence or character.

The latter two types of genetic engineering are of interest only insofar as they affect social and ethical issues and the public perception of gene therapy. The history of eugenics is long and complex (see Kevles, 1985); due to its practice in Nazi Germany it is rightly feared as a means of repression and control. All human traits encompassed by the term eugenics, including intelligence, personality, character, and creativity, are felt to be governed by complex multigenic interactions (to the extent that such traits are inheritable). On the other hand, single gene deficits resulting in mental retardation or personality disorders such as phenylketonuria or Lesch-Nyhan syndrome are appropriate candidates for gene therapy.

Genetic enhancement is fraught with technical difficulties. It also raises major ethical considerations. With our current level of knowledge, insertion of new genetic information may result in harmful effects (eg, insertional mutagenesis) as well as potential benefits. In addition, many genes are tightly regulated or are parts of complex metabolic pathways and insertion of additional genetic material may have many unknown and unanticipated detriments. Thus it is not anticipated that gene therapy will address either genetic enhancement or eugenics in the near future. Social and ethical issues are discussed in greater detail in Chapter 11.

Germline gene therapy has the theoretical advantage of permanently curing a genetic disease. Of course certain genes (eg, very large genes such as the Duchenne's muscular dystrophy gene, dystropin) have a naturally high mutation rate that would not be affected by this process. If the manipulation could be performed early enough in the individual's life, neither the patient nor his or her offspring would suffer from the genetic deficit. Such therapy might be attempted by injection of the corrected gene directly into a fertilized egg at risk. This technology is widely used for generation of transgenic animals (for details on germline gene manipulation and transgenic animals, see Chapter 8). At our current level of genetic understanding and technical ability, however, such "therapy" might well result in harm more often than in benefit. Microinjection of genes into tissue culture cells can be accomplished with reasonable efficiency (some 20% of cells injected can be permanently transfected), however, there is a high failure rate. In recent work only 20% to 30% of microinjected murine eggs survive to live birth of an individual carrying the gene. Microinjection of genes into eggs or early embryos may have potentially deleterious results because we have no means to control where the injected DNA will insert itself in the genome. Insertion of the gene into totipotent stem cells may ultimately result in its expression in inappropriate tissues. As an example, it is unknown how the expression of a gene normally expressed in lymphocytes, such as the adenosine deaminase gene, might affect muscle cells. The exogenous gene may integrate within a normal gene resulting in dysfunction or ablation of that gene. Conceivably, exogenous DNA combined with a controlling element might integrate near a proto-oncogene resulting in eventual neoplastic transformation. Finally, microinjection of replacement genes into defective eggs would be of limited usefulness. It is antici-

pated that replacement gene therapy would be most appropriate for autosomal or sex-linked recessive mutations where loss of the normal gene product results in disease. For many dominant mutations expression of an abnormal gene is thought to result in manifestations of disease; for such disorders gene replacement or gene modification will be necessary, procedures that are technically much more difficult than addition of a gene (see below and Chapter 3). For autosomal recessive mutations, only 25% of fertilized eggs are at risk whereas for X-linked recessive mutations, only 50% of male fertilized eggs (again 25% of total eggs) are at risk to develop the disease. Since at our current level of sophistication it is difficult to determine which fertilized eggs (or even early embryos) are at risk, 75% of normal eggs manipulated by microinjection would actually be at risk for a deleterious outcome. This result is not acceptable. For these reasons, it is not anticipated that germline gene therapy will be attempted in humans in the foreseeable future.

Genetic manipulation of embryos falls somewhere between manipulation of fertilized eggs and manipulation of somatic tissue in a fully developed adult, a child, or even a fetus. The embryo still possesses many pluripotent "stem" cells compared to the number of terminally differentiated cells. It is conceivable that the result of a somatic genetic manipulation would find its way into the germinal tissue and be passed on to subsequent generations. It is possible that embryos might be assayed for the presence or absence of a genetic defect before they were subjected to gene therapy; for example, by separating a small number of cells from the embryo, growing them in culture, and conserving the rest of the embryo until the results of assays became available. Nondefective embryos could be reimplanted directly while those embryos containing genetic disorders could undergo gene therapy prior to reimplantation. Such separation and culture of embryos is at present socially unacceptable although it may be within our technological capabilities. In addition, application of this technique requires embryo transfer, a procedure used now in conjunction with in vitro fertilization (see Chapter 9). Currently, the pregnancy rate following embryo transfer is 10% to 15% with approximately half of these pregnancies ending in viable birth. These results would severely limit the chances of successful gene therapy in human embryos.

Despite our current inability to manipulate fertilized eggs or embryos, it is possible that the technology and legal sanctions will become available in the not-too-distant future. Such a capability will raise a number of important social and ethical issues that deserve consideration prior to employment of the technology (see Chapter 11).

Somatic cell gene therapy refers to manipulation of body cells other than germinal tissue and is the type of manipulation most commonly referred to by the general term "gene therapy." Somatic cell gene therapy is performed on fully developed individuals. It is anticipated that the most suitable patient will be an infant or child with a proven genetic disorder; however, a place may exist for initiating gene therapy at the fetal stage, particularly if the disorder involves the central nervous system. Somatic cell gene therapy has the theoretical advantage of application at virtually any stage of development. Any tissue potentially is amenable to manipulation although technical considerations suggest that those tissues with a significant capacity for self-renewal have the best prospects for permanently incorporating exogenous genetic material. Experiments using somatic cells do not place the whole organism at risk should they fail. Only parts of organs or tissues need be employed and because not all tissue is used for any single manipulation, the experiments can be repeated. Gene transfer techniques need not be 100% efficient to be of

significant benefit to the treated individual. The disadvantage is that the correction cannot be passed on to the individual's progeny. Even so, if gene therapy allows the patient to survive to reproduce and the next generation carries the defect, the overall frequency of the defective gene will not increase significantly in the population since the vast majority of defective alleles are transmitted by heterozygote carriers. For example, if a recessive lethal mutation exists in a population at an allele frequency of 1%, 1 out of 50 people (1 out of 100 chromosomes) will carry the gene, but only 1 of 10,000 will be homozygous for the defective gene.

The diseases and tissues that will prove amenable to somatic gene therapy will be determined only by further study of specific diseases and techniques. What considerations should go into the design of experiments for human gene therapy (Table 1.4)? An important consideration is to choose an appropriate disease. Current technological considerations suggest that single gene disorders of recessive inheritance, ie, those with a defective or missing enzyme or protein, are the most appropriate with which to begin. Because of uncertainties in the success of early procedures, only diseases with significant morbidity or mortality should be treated such that potential benefits outweigh risks. Current therapy must be inadequate or nonexistent. The most successful experiments would be anticipated to involve defects in tissue that is accessible to experimental manipulation; for example, diseases involving bone marrow cells (see Chapter 6) are a particularly attractive target. The potential of gene therapy for treatment of particular human diseases is discussed in more detail in Chapter 8.

Preliminary experiments in vitro are necessary. The gene causing the defect

Table 1.4. Considerations for Experimental Design and Implementation of Human Gene Therapy

I. *Appropriate disease*
- Single-gene disorder, recessive inheritance
- Significant morbidity or mortality
- Current therapy inadequate or unavailable
- Accessible cellular site of genetic defect causing phenotype

II. *Preliminary experiments in vitro or in experimental animals*
- Obtain clones capable of transducing active gene product
- Demonstrate efficient gene transfer and appropriate expression in target cells in vitro and in vivo
- Evaluate side effects in live animals (malignancy, mutagenesis, metabolism)
- Demonstrate efficient gene transfer into human cells in vitro
- Demonstrate revision of pathologic phenotype in appropriate animal model if available

III. *Design of experimental protocol for human experimentation*
- Assess risk/benefit of proposed therapy compared with conventional therapy
- Assess risk of vertical (germline) or horizontal (infectious) transmission of vector
- Informed consent from patient or guardian
- Assess impact on individual and family
- Assess plans for disseminating information to public

IV. *Obtain approval for human experimentation*
- Proposal reviewed by Institutional Review Board and Institutional Biosafety Committee
- Proposal reviewed by Recombinant Advisory Committee after publication of precise and public comment
- Proposal reviewed and approved by director of National Institutes of Health

(Adapted from Ledley, 1987.)

must be cloned and be available in a configuration that will allow efficient gene transfer and expression in appropriate target cells. The target cell for gene transfer should ideally have the capacity for self-renewal and be able to differentiate into all cell lineages that need to express the gene to alleviate disease symptoms. This will ensure long-lasting effects of gene therapy. In particular, efficient transfer and expression in human target cells in vitro should be shown prior to attempts to treat patients. Diseases with reasonable animal models, where efficacy (ie, reversion of the pathologic phenotype) can be demonstrated are optimal ones to investigate for gene therapy. In such cases, evaluation of possible side effects such as mutation, interference with normal metabolism, and potential for malignancy can be made. Gene transfer studies in animals, both mice and larger mammals, are essential before serious human gene therapy trials begin. Early attempts at gene therapy in humans will be most productive with diseases where partial correction of the enzyme deficiency will be sufficient for alleviation of clinical symptoms. Early experimental protocols must assess the risk/benefit ratio of the proposed treatment compared to no therapy or conventional alternatives. The risk of germline (vertical) transmission and, also, infectious (horizontal) transmission of the repaired gene or its associated vector must be assessed. The impact of treatment and potential complications for the patient and his or her family must be evaluated and informed consent obtained. Finally, plans for disseminating reliable and understandable information to the public must be made. Before any gene therapy experiments can be undertaken, review by institutional review boards and biosafety committees will be required. Federally supported clinical trials also will require the approval of the director of the National Institutes of Health (NIH).

Which particular diseases might be most amenable to somatic cell gene therapy? Because the most simple method appears to be addition of new genetic material to bone marrow cells, recessive diseases involving primarily bone marrow are the logical candidates (see Table 1.5). Such diseases include deficiencies of the enzymes adenosine deaminase and purine nucleotide phosphorylase, both of which result in severe combined immunodeficiency. In addition, disorders of hemoglobin such as thalassemia or sickle cell anemia might be amenable to treatment. Disorders of serum proteins produced normally by cells other than bone marrow might be rectified by therapy of bone marrow cells if the proteins produced can be released from these cells into the serum. Potential candidates include hemophilia (factor VIII or IX deficiency), α_1-antitrypsin deficiency, and deficiencies of circulating complement factors. Bone marrow storage diseases resulting from defective metabolic enzymes, such as glucocerebrosidase deficiency (Gaucher's disease) or galactosidase deficiency (Fabry's disease), are candidates. Diseases where the tissue predominantly involved is not bone marrow present more of a challenge to present technology. Theoretically, any tissue with regenerative capacity (implying the existence of stem cells capable of persistent cell division) might be treatable using somatic gene therapy. These disorders might include inborn errors of metabolism involving liver enzymes such as phenylalanine hydroxylase (phenylketonuria) or urea cycle enzymes (eg, carbamyl phosphate synthetase and argininosuccinate lyase); deficiency of low-density lipoprotein receptors; endocrine disorders such as deficiencies of insulin, growth hormone, or parathyroid hormone; and muscle diseases such as Duchenne's muscular dystrophy. Progress using hepatocytes, skin fibroblasts, myocytes, and keratinocytes as target cells has been reported. Diseases involving the central nervous system where (a) there is no known regenerative compartment and where (b) damage to neurons might be present before birth, such as hypoxanthine