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On the Molecular Genetics of Retinitis Pigmentosa

Peter Humphries, Paul Kenna, G. Jane Farrar

The human retina carries specialized neurons, the rod and cone photoreceptors, which absorb and transduce light energy and transmit impulses through the optic nerve to the brain. The most prevalent group of inherited retinopathies, affecting approximately 1.5 million people, is collectively termed retinitis pigmentosa (RP). Mutations responsible for RP have now been found in two genes encoding transmembrane proteins of the rod photoreceptor outer segment disc, and a number of additional causative genes have been localized. It is likely that characterization of the majority of such genes over the next few years will lead to a substantial elucidation of the molecular pathology of this debilitating group of hereditary conditions.

The term retinitis pigmentosa (RP) is used to describe a genetically and clinically heterogeneous group of human inherited retinopathies. These debilitating disorders collectively represent the most frequent inherited forms of human visual handicap, with an estimated prevalence of approximately 1 in 3000 (*1, 2*). RP may be inherited autosomal dominantly (adRP), autosomal recessively (arRP), or in an X-linked fashion (xlRP). A number of studies have estimated the relative proportions of these various forms of RP (*3*). Typically, arRP together with single isolated cases seems to be the most frequent, representing at least half of the families affected by the disease. In contrast, on average both adRP and xlRP are present in between 10 to 15% of RP families. However, estimates vary between studies and are affected by the extent of consanguinity, by ascertainment biases, and so on.

In the past 5 years many of the technical developments in human molecular genetics have been applied successfully in the study of RP and have led to a greater understanding of the underlying mechanisms of pathogenesis in this group of disorders. The rapid emergence of a genetic map of the human genome has facilitated the localization of genes responsible for some forms of RP. The development of polymerase chain reaction (PCR) technology, together with the identification of many highly polymorphic genetic markers based on simple sequence repeats, has speeded the localization of disease-causing genes. Moreover, the development of methods for rapid identification of sequence variation, such as single strand conformational polymorphism electrophoresis (SSCPE) (*4*), heteroduplex analysis (*5*), denaturing gradient electrophoresis (*6*), and direct sequencing of amplified DNA products (*7*), has provided a means of investigating genes that are considered to be "candidates" for a particular disease. As a result of such technical developments two xlRP genes have been localized to the short arm of the X chromosome (*8*). Furthermore, three adRP genes have been mapped: to the long arm of chromosome 3 (*9, 10*), to the short arm of chromosome 6 (*11, 12*), and to the pericentric region of chromosome 8 (*13*). More recently, mutations within the rhodopsin gene, which maps to 3q (*14*), and the peripherin/*RDS* gene (*15, 16*), which maps to 6p, have been implicated as causative in some forms of adRP. Both of these genes encode proteins that traverse the outer segment disc membranes of the rod photoreceptor cells. Thus, we have begun to elucidate the cause of some forms of this large group of retinopathies. However, although we have made great strides in this direction, as yet many of the genes involved in RP have not been localized. Moreover the means by which mutant rhodopsin and peripherin proteins cause photoreceptor degeneration remain unknown. In this review we will discuss the recent developments in the field and the predicted future directions that research will need to take to address these important questions.

RP is a degenerative disease characterized initially by the development of night blindness, or nyctalopia, owing to the progressive death of the rod photoreceptor cells. Long before the outward signs of RP develop however, electrophysiological changes may be detectable in the retina. A test to screen for such changes is called an electroretinogram, or ERG. This measures the electrical activity generated by the retina in response to flashes of light. Although first discovered by the Swedish physiologist Holmgren in 1870 (*17*), it was not until 1945 that Karpe (*18*) documented that the ERG response was abnormal in patients with RP. Such abnormalities are usually recordable even in presymptomatic cases. Rod responses are reduced in amplitude but may be normal or delayed in timing (Fig. 1). Cone responses may also be affected, wave forms being abnormal in amplitude or timing or both. In advanced cases of the condition all responses are extinguished. This may be the case even where central vision is relatively well preserved.

The death of the rod cells precipitates more extensive tissue degeneration (Fig. 2). Cone cells begin to die off and the vessels supplying blood to the retinal membranes become attenuated. As a result, daytime vision becomes noticeably affected. The retinal epithelium itself becomes thinner, and as the pathological changes develop, deposits of pigment often build up on the retinal surface, the latter phenomenon accounting for the name of the disease. The visual fields become constricted, and affected patients develop tunnel vision and often lose all effective sight. [For comprehensive clinical reviews, see (*3*) and (*19*).]

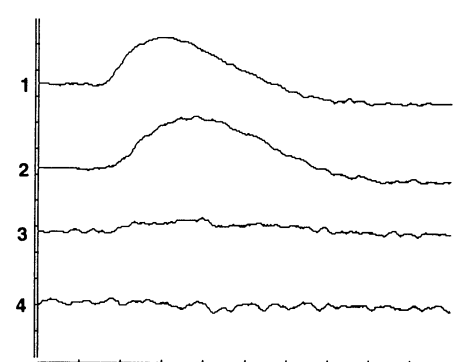

Fig. 1. Shown here are rod responses from a normal individual and from RP patients. Tracing 1 is from a normal individual. Tracings 2 and 3 demonstrate reduced amplitude and delayed responses from RP patients. Tracing 4 shows an extinguished response from a patient with advanced RP. The horizontal time base is 30 milliseconds per division. The vertical amplitude axis is 250 μV per division for tracing 1 and 100 μV per division for all other tracings.

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RP is at present unpreventable and incurable. A prerequisite to the development of any form of preventive therapy is a knowledge of the cause of a disease. Yet, in the case of RP, nothing was known of the molecular events that bring about the death of the rod cells. Could the defect lie in the visual transduction cycle itself, the cycle through which photons impinging on the rod cell are transduced into an electrical impulse that is transmitted to the brain? Alternatively, could the problem lie in some defect of vascularization resulting in a reduction of vital blood supply to the retinal cells? Many other potential causes are plausible.

A number of approaches could lead to the identification of genes responsible for RP. First, the technique of genetic linkage analysis could be deployed. This method, which has proven to be of great success in localizing genes for a number of hereditary conditions (such as Huntington's chorea, cystic fibrosis, neurofibromatosis, myotonic dystrophy, and so on), relies on mapping the disease gene with the use of a variety of genetic markers in families segregating various forms of the disease (*20, 21*). RP is a hereditary condition, and many large families, particularly with X-linked and autosomal dominant forms of the disease, are available for study. Second, a "candidate" gene approach could be employed to search for genes that could be involved. Hunting for mutations in such genes, in DNA from affected patients, might result in the identification of disease-causing mutations. Over the last 5 years or so, both approaches have been used, and with considerable success.

The Mapping of Retinitis Pigmentosa Genes

The first genetic linkage studies to be carried out in RP were reported by Bhattacharya and colleagues in 1984 (*22*). They established a linkage between the segregation of a polymorphic DNA marker named L1.28 (DXS57) and RP in a series of five families showing X-linked forms of the disease (in this form of the disease RP is never transmitted from male to male). The position of L1.28 on the X chromosome had previously been determined by the process of in situ hybridization in which the radiolabeled DNA marker was allowed to hybridize to its homologous sequence on the intact chromosome. Its position had also been determined by mapping studies with the use of a series of somatic cell lines containing overlapping pieces of the X chromosome (*23, 24*). The marker, and hence the RP locus, mapped to the proximal short arm of the chromosome quite close to the centromere. These data turned out, in fact, to be slightly at odds with map locations derived for the RP locus from studies of patients with X chromosome deletions. Several patients were known, each of whom suffered from a variety of X-linked conditions, who had extensive deletions of the short arm of the X chromosome as the molecular basis for their disease (*20*). Three patients in particular (BB, OM, and SB) proved to be of great value in pinpointing the location of an RP gene. BB suffered from a combination of three sex-linked disorders, the Duchenne form of muscular dystrophy, chronic granulomatous disease (CGD), and RP. He also lacked the red cell antigen called Kx. SB, on the other hand, also lacked Kx but suffered from only CGD and RP. The third patient, OM, lacked the Kx antigen but suffered from only one of the aforementioned diseases, namely CGD. Because the positions of the deletions causing these diseases could be accurately determined by physical mapping studies and cytogenetics, a precise location for the RP locus could be worked out (Fig. 3). The RP gene resides in the tiny region of proximal overlap of these deletions. Genetic linkage studies initially placed the RP locus, closer to the centromere than the locations of overlap of the deletions described above. The disparity was substantial, being of the order of perhaps ten million base pairs or so. As more genetic linkage data were accrued, however, the paradox resolved itself. On a study of pooled linkage data from 62 X-linked RP pedigrees from many countries, Ott and colleagues were able to confirm what many investigators had suspected for some time, that is, the presence of two RP loci on the X chromosome (*8*). One locus (RP2) maps between the marker p58.1 (DXS14) and the centromere, and the second (RP3), as localized by the deletion studies, maps somewhat more distally (Fig. 3). Neither gene has yet been characterized.

Mutations in the Rhodopsin and Peripherin *RDS* Genes

A number of systematic genetic linkage studies, designed to determine the location of a disease-causing gene by establishing co-segregation between the disease phenotype and a polymorphic genetic marker, have been carried out over the last few years. The first adRP gene was located in 1989 as a result of extensive work with a large family from Ireland. The family, termed pedigree "M," had over 50 affected persons who were suffering from a particularly early onset form of disease, in which night vision problems (nyctalopia) were

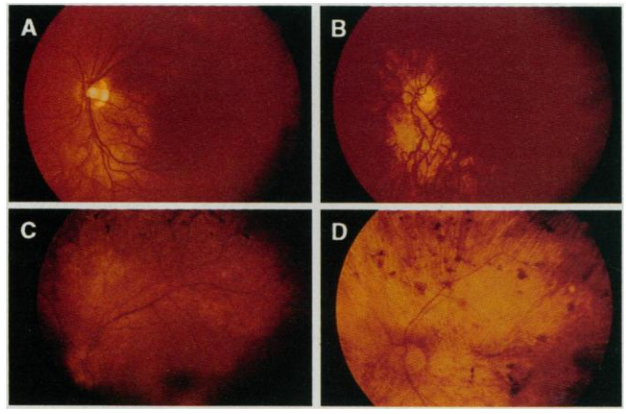

Fig. 2. (**A**) Normal optic disc, retinal blood vessels, and macula. (**B**) Fundus photograph from an 8-year-old member of a kindred affected with adRP. Note the marked thinning of the retinal pigmented epithelium in the region of the optic disc and the attenuation of the retinal blood vessels. (**C**) Mid-peripheral "bone corpuscle" pigment deposits from which RP derives its name. (**D**) Advanced degenerative changes, including pallor of the optic disc, attenuation of the retinal blood vessels, marked thinning of the retinal pigmented epithelium, and clumps of pigment deposits.

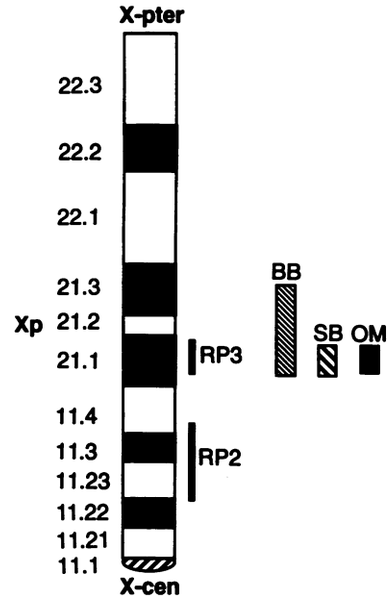

Fig. 3. Schematic drawing of the short arm of a human X chromosome, indicating the approximate locations for RP2 and RP3. RP2 has been localized by linkage studies only. By contrast, RP3 has been mapped both by linkage studies and with patients who carry deletions. The three key deletions (BB, SB, and OM) that have aided in the localization of RP3 are indicated.

present essentially from birth and severe visual handicap invariably resulted by the second decade of life. After coverage of large sectors of the genome, a gene was eventually located on the long arm of chromosome 3 close to the DNA marker C17 (D3S47) (9). This observation provided an immediate clue as to the possible cause of the disease, as the gene encoding rhodopsin had recently been mapped on the long arm of this chromosome (*25*). A genetic marker had recently been identified within the rhodopsin gene and was subsequently used by Farrar and colleagues in a multilocus analysis with other markers on the long arm of the chromosome (*10*). It turned out that rhodopsin mapped between the markers C17 and L.182, and none of these markers showed any recombination with the RP disease locus. Moreover, in this analysis rhodopsin was shown to be linked to the disease locus with a lod score of approximately 20, the highest lod score ever attained by genetic linkage analysis of a single pedigree. (A lod score, a logarithm of the odds ratio, is a measure of the likelihood of linkage, as opposed to nonlinkage, given a particular set of data. Lod scores of 1, 2, and 3, for example, provide odds of 10, 100, and 1000 to 1 in favor of linkage. The observed lod score of 20 thus provided an exceedingly strong indication of linkage for the disease to that locus in the Irish pedigree.)

These observations fueled the search for mutations within the rhodopsin gene in patients with adRP. The first mutation was identified by Dryja and colleagues in 1990 (*14*). The mutation was an amino acid substitution at codon 23 of the coding sequence, which resulted in the replacement of proline with histidine (Pro-23-His). The mutation was common in adRP patients in the United States, accounting for up to 15% of all cases of the disease. However, in a large-scale survey of over 100 autosomal-dominant patients in the populations of Europe, the mutation was found to be absent (*26*). Thus, the prevalence of the first, and most common, dominant RP mutation may have been the result of a founder effect in the U.S. population. Well over 30 mutations within the rhodopsin gene have now been identified in cases of adRP (*21*). Most are point substitutions in conserved regions of the molecule. A number of small deletions and a couple of frame-shift mutations that result in a radical alteration of the carboxyl terminus of the protein have also been encountered.

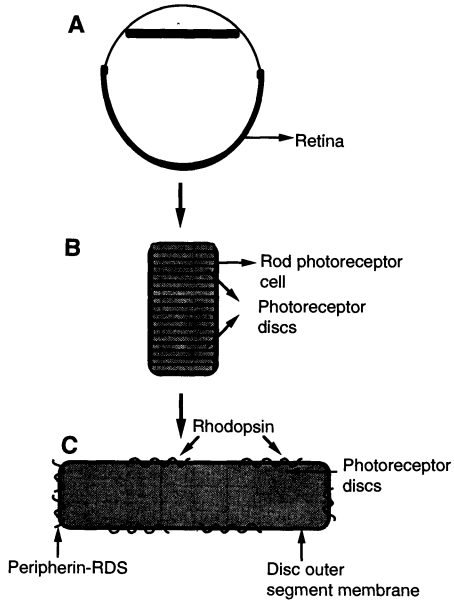

Fig. 4. Diagrammatic representations of the eye (**A**), indicating the neural retina; of an individual rod photoreceptor outer segment (**B**), showing numerous stacked discs within the outer segment; and of a single disc (**C**), with rhodopsin and peripherin-RDS molecules weaving in and out of the disc membrane.

Shortly after the establishment of the first autosomal dominant linkage it became apparent that only a minority of families showed rhodopsin involvement. A second large family of Irish origin, for example, provided no evidence for linkage on 3q (*10*). Another gene had to be involved. Other families with no linkage on 3q were rapidly identified (*27, 28*). The Irish family (the pedigree is termed "G/M") showed much later onset of RP than did the first rhodopsin-linked pedigree, with night vision problems and field loss not developing until the fourth decade and beyond. Continued genetic linkage studies localized the causative gene on the short arm of chromosome 6, close to the major histocompatibility locus (*11, 12*). Again, as in the first study, the region of linkage contained a candidate gene. This was the gene which encodes the protein, peripherin *RDS*, a structural component of the rod cells. For some time it has been known that a defective form of peripherin *RDS* is involved in the cause of the naturally occurring retinopathy of the mouse called *rds* (retinal degeneration slow) (*29, 30*). Peripherin *RDS* resides in the membranes of the outer segment discs of the rod cells, as does rhodopsin (Fig. 4). Unlike rhodopsin, however, peripherin *RDS* does not absorb light. It is located at the periphery of the discs, and its major, and possibly only, function is believed to be one of maintaining the structure of the disc membranes (*31*). Mice heterozygous for the *rds* mutation produce distorted and vacuolated discs, whereas those animals homozygous for the mutation fail to produce rod outer segment discs at all. After the establishment of linkage, Farrar and colleagues reported a mutation in the peripherin *RDS* gene in a patient with a dominant form of RP. The mutation cosegregated with the disease in the pedigree in which it was identified and was not encountered in the non-affected population (*15*). This mutation is a trinucleotide deletion that removes one of a pair of cysteine residues at codons 118 and 119 of the protein. In parallel candidate gene studies, three additional mutations within the *RDS* gene were observed by Kajiwara and colleagues in DNA from patients with adRP (*16*).

How do mutations in rhodopsin and peripherin *RDS* cause RP? Does phototransduction have any direct relevance to disease causation? Rhodopsin's function as a light-absorbing pigment might, in many instances, be coincidental. Evidence now suggests that some mutant rhodopsins may not be effectively transported from the endoplasmic reticulum after translation (*32*). Such aberrant transport may, by some as yet unknown mechanism, initiate the pathological processes that result in rod cell mortality. Moreover, those mutant proteins that are transported to the outer disk membranes may result in membrane destabilization. Mutant peripherin *RDS* proteins might exert their effects in either of the above ways. Now that efficient expression systems are available for production of large quantities of biologically active mammalian proteins (the baculovirus expression system, for example), it should be possible to investigate and compare the functional and structural activities of normal and mutant proteins in considerable detail, and hence gain insights into underlying pathological mechanisms.

An additional locus for adRP has also been identified in the pericentric region of chromosome 8 (*13*), although no candidate gene has been identified in this region. Moreover, we now know, as a result of continued genetic linkage studies, that additional pedigrees exist with adRP that appear to show no evidence for linkage in the regions of known genes (*33*). Hence, at least one more dominant locus remains to be identified. Whether all gene products in this form of the disease localize to the outer segment disc membranes remains to be determined.

In principle, genetic linkage analysis could readily be applied to arRP. In practice, however, such families are generally insufficiently large to enable realistic linkage work to be undertaken, and pooling data from multiple pedigrees would be unwise in view of the likely genetic heterogeneity. Occasionally, however, large pedigrees with arRP have been identified. One such family, for example, resides in the Netherlands, close to Amsterdam, and has approximately 40 persons currently suffering from a form of arRP. Although no systematic genetic linkage studies have yet

been reported in this family, both the rhodopsin and peripherin *RDS* loci have been excluded (*34*). Exclusion of the rhodopsin gene is of interest in that in a patient with arRP from the United States, a null rhodopsin mutation has recently been detected (*35*). The individual in question is homozygous for a nonsense mutation at codon 249 of the gene (GAG mutated to TAG). This is predicted to result in an absence of the fourth and fifth transmembrane domains of the rhodopsin molecule. Absence of a linkage to the rhodopsin locus in the Dutch pedigree demonstrates the existence of genetic heterogeneity in this form of the disease.

Another interesting concentration of families with arRP resides in the state of Louisiana. These families suffer from Usher syndrome, a recessive condition involving both deafness and RP. Usher syndrome is broadly divisible into two categories, type I and II: type I is associated with more profound hearing and balance problems than type II. Almost 200 nuclear families affected by Usher syndrome have been identified in southwestern Louisiana among the Acadian community. Both disease types have been observed, suggesting that more than one gene may be segregating (*36*). However, recent studies by Kimberling and colleagues on eight type II families gathered from the United States and Europe have resulted in the establishment of a genetic linkage in this form of the disease to a series of markers on the long arm of chromosome 1 (*37*). However, the causative gene remains to be precisely localized.

A Multitude of Potential Retinopathy Genes

Rod photoreceptor cells are highly specialized structures. Each cell is divided into two compartments, the inner and outer segments. The former compartment holds the machinery necessary for the synthesis of proteins. It is within the outer segments that light is absorbed and the process of visual transduction, that is, the conversion of photons of light into an electrical impulse, takes place (*38, 39*). Electron microscopy of the outer segments reveals a series of stacked discs (Fig. 4). The photoreactive pigment, rhodopsin, composed of an opsin molecule bound to the chromophore 11-*cis* retinal, is a transmembrane component of the discs. The amino terminus of the protein resides within the lumen of the disc, whereas the carboxyl terminus is in the cytoplasm. Absorption of a photon of light results in the activation of the molecule by isomerization of 11-*cis* retinal to the all-*trans* form (Fig. 5). Activated rhodopsin is able to interact with the G protein transducin to catalyze the exchange of GDP on the G protein's surface to GTP. In this process, the alpha subunit of transducin, charged with GTP, dissociates from the beta and gamma subunits. In this primary step the visual signal is amplified considerably, because one activated rhodopsin molecule is able to activate up to 500 transducin molecules. The activated alpha subunit of transducin activates, in turn, the enzyme cyclic GMP phosphodiesterase (PDE). This enzyme consists of four subunits, alpha, beta, and two gammas. Activation is achieved by displacement from the enzyme of the two gamma subunits. Activated PDE hydrolyzes cGMP, which is bound to the rod photoreceptor cGMP-gated channel protein, a transmembrane protein that allows monovalent and bivalent cations to move across the rod cell membrane. At least three, probably more, cGMP molecules are bound to each channel protein. Their hydrolysis results in a closure of the ion channels and leads to hyperpolarization of the rod cell plasma membrane. This initiates the visual transduction signal. Calcium ions, however, continue to be pumped out of the cell by a glycoprotein sodium-calcium exchanger. Removal of calcium results in the activation of the protein recoverin, which activates the enzyme guanylate cyclase that in turn restores the quantity of cGMP in the cell and permits a reopening of the cGMP-gated channels. Rhodopsin, in the meantime, is deactivated as a result of phosphorylation in the region of its carboxyl terminus by the cooperative action of the enzyme rhodopsin kinase and a 48-kilodalton protein called arrestin, or S-antigen.

Why should we suppose that mutated versions of any of these proteins could cause the death of rod cells in RP? A mutation in one of them is known to cause an inherited retinopathy of mice, which in many respects resembles RP in humans. The mouse retinopathy is called retinal degeneration (rd), and it is caused by a defect in the beta subunit of cyclic GMP PDE (*40, 41*). Thus, any of the above proteins could be potential candidates for the cause of RP in humans, provided that expression of these mutant proteins, perhaps in cells other than photoreceptors, is not lethal. With the development of rapid mutational screening techniques based on the PCR, the possible involvement of such genes in the etiology of inherited retinopathies can now be explored in detail.

Conclusions and Prospects

Recent technical developments in the field of molecular biology have been successfully deployed in the study of the group

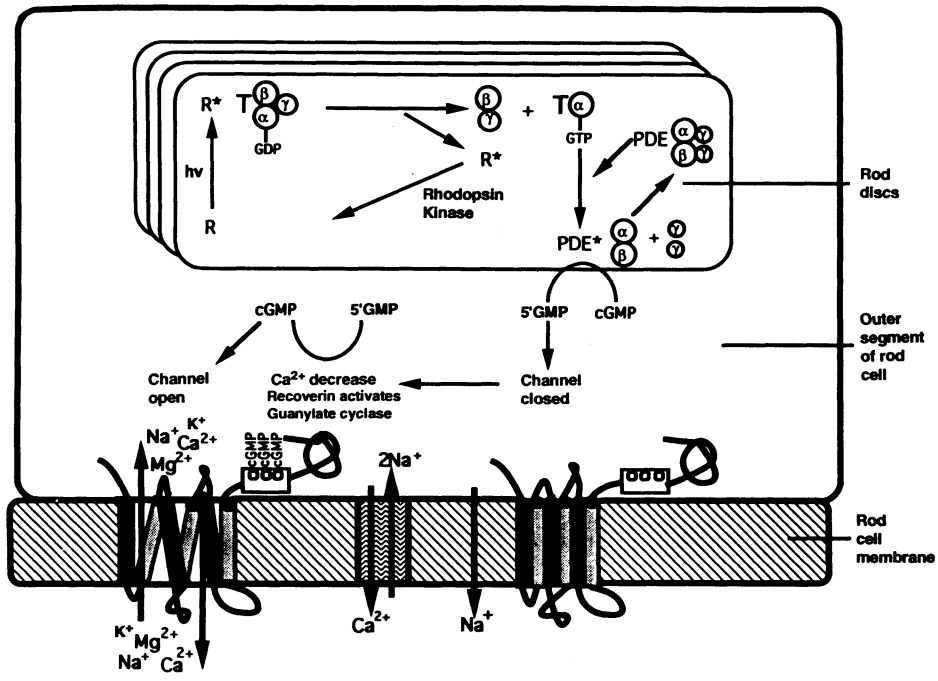

Fig. 5. Some key steps in visual transduction (*38, 39*). Photoactivated rhodopsin molecules stimulate transducin, which activates cGMP phosphodiesterase, leading to reduced cytoplasmic cGMP concentrations and hence closure of cGMP-gated channels. Lowered calcium levels stimulate recoverin and guanylate cyclase activities and result in cGMP production. The rod cell is returned to the "unexcited" state due to (i) a rise in cGMP concentrations that reopens the cGMP-gated channels and (ii) the phosphorylation of photoactivated rhodopsin by rhodopsin kinase. R, rhodopsin; R*, photoactivated rhodopsin; T, transducin; PDE, phosphodiesterase; and PDE*, activated phosphodiesterase.

of debilitating inherited human retinopathies termed RP. In the past few years, both linkage and candidate gene studies have been used to localize a number of disease-causing genes and, in some instances, identify specific mutations within candidate genes.

One can use prior knowledge of the biochemistry of visual transduction to predict genes that potentially may be involved in human retinopathies and hence direct future linkage and candidate gene studies. The genes encoding transducin, rhodopsin kinase, arrestin, and interstitial retinol binding protein are just a few of many potential candidates. It seems likely, for example, that the beta subunit of PDE, which has been shown to be responsible for the retinopathy observed in the *rd* mouse, may be involved in RP or in an RP-like retinopathy. Given the rapid techniques now available for such studies, it is probable that the majority of genes involved in RP will be characterized within the next few years. However, although great strides have been made toward identifying the genes responsible for RP, little as yet is known about the means by which the mutant proteins encoded by these genes cause photoreceptor cell death. Harnessing of expression systems to produce large quantities of biologically active proteins will enable investigation of the activities of normal and mutant proteins, such as rhodopsin and peripherin *RDS*, and may thereby shed light on pathogenic mechanisms in RP. Furthermore, the powerful technique of targeting potential retinopathy genes by homologous recombination will enable identification of more disease-causing genes and investigation of the course of the retinal degeneration observed in the chosen animal model. As research diversifies, we will not merely elucidate the molecular pathology of this group of distressing conditions but will also gain a greater understanding of the functions of these proteins in the healthy retina.

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Human Gene Therapy

W. French Anderson

Human gene therapy is a procedure that is being used in an attempt to treat genetic and other diseases. Eleven clinical protocols are under way at the present time, each with scientific and clinical objectives. Human genetic engineering raises unique safety, social, and ethical concerns.

Human gene therapy has progressed from speculation to reality in a short time. The first clinical gene transfer (albeit only a marker gene) in an approved protocol took place on 22 May 1989, almost exactly 3 years ago; the first federally approved gene therapy protocol, for correction of adenosine deaminase (ADA) deficiency, began on 14 September 1990. Now there are 11 active clinical protocols (Table 1) on three continents with nine more approved protocols about to begin and over a dozen additional protocols in various stages of development.

What are the objectives of these protocols? What are the scientific and clinical questions that they are asking? How safe are they? These are the questions that this review addresses. The extensive preclinical studies that support each clinical protocol are not covered.

An unsuccessful attempt was made in 1980 to carry out gene therapy for β-thalassemia with the use of calcium phosphate–mediated DNA transfer. Retroviral-mediated gene transfer was developed in the early 1980s in animal models (*1*). This technology is the principal procedure used today. Many recent reviews on retroviral vector development, packaging cell lines, and alternate gene delivery techniques (*2*) as well as on gene transfer and expression in cell cultures and in animal models (*3*) address the technical issues upon which human gene therapy relies.

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NeoR/TIL Gene Marking

The first federally approved human genetic engineering experiment, initiated in 1989, was for the transfer of gene-marked immune cells (specifically, tumor-infiltrating lymphocytes, or TIL) into patients with advanced cancer. The protocol had two primary objectives: (i) to demonstrate that an exogenous gene could be safely transferred into a patient and (ii) to demonstrate that the gene could be detected in cells taken back out of the patient (*4*).

The protocol asked a number of scientific and clinical questions, generated by several earlier experiments. In 1986, a clinical protocol for the treatment of advanced malignant melanoma with the newly dis-