Gene therapy has been proposed as a radical means of providing a permanent cure for inherited diseases. It involves replacing or complementing the defective gene in the cells of a patient with its artificially introduced functional counterpart.

Clinical/molecular interface

In her opening remarks, the President of the College, Professor Margaret Turner-Warwick, stated that the aim of the meeting was to provide a scientific-clinical interface allowing the exchange of ideas between the scientific and medical communities.

In his introductory lecture Sir David Weatherall FRS (Institute of Molecular Medicine, Oxford) provided a general overview of current prospects for gene therapy. Although it has arrived at a promising stage, he suggested that gene therapy is not imminent. More than 4,000 single gene disorders are currently known but, despite advances in our ability reliably to diagnose a number of monogenic disorders, the treatment regimens available for the majority of these conditions remain for the most part inadequate. A recent survey of 351 different monogenic disorders revealed that in 48% of these conditions the symptomatic treatment offered is of no benefit to the long-term survival prospects of the patient; in only 12% can treatment be regarded as successful. Overall, it can be concluded that traditional symptomatic treatment is not effective in the majority of cases. The arguments for a radical alternative in the form of gene therapy become more compelling in the light of these findings.

Two forms of gene therapy are possible: somatic cell or germ cell gene therapy. Currently, only the former can be considered as a viable option. Germ cell therapy with its implications for the transmission of genetically engineered 'improvements' to future generations is ethically unacceptable and it would be negligent even to consider the use of germ cells before the long-term risks of gene therapy in somatic cells have been properly evaluated.

Several criteria are prerequisites for gene therapy to be considered as treatment for a particular genetic disease. First, the disease must be fully understood both genotypically and phenotypically. One further requirement is that any tissue-specific and developmental modes of gene expression have been fully characterised. Another consideration is that, whilst housekeeping genes are able to tolerate some degree of flexibility in expression levels, this does not hold for the majority of tissue-specific genes. This is well illustrated by the finding that β-thalassaemia is caused as much by the relative over abundance of α-globin chains, created as a consequence of the reduced levels of β-globin in the red cells of thalassaemia patients, as by the reduced β-globin levels per se. Cellular phenomena such as parental 'imprinting' and X-inactivation must also be taken into consideration as factors potentially able to influence expression levels of the introduced gene. Another factor determining the feasibility of gene therapy is accessibility of the chosen target tissue for in vitro manipulation and subsequent retransplantation into the patient. Some disorders could conceivably be treated by gene therapy using a tissue other than that in which it is normally expressed; however, this is not always the case. For instance, whilst manipulation of skin cells is a relatively non-invasive and therefore therapeutically attractive procedure, this cell type would be inappropriate if gene therapy was being considered as an option for the treatment of neurological disorders. Once the manipulated cells are retransplanted into the patient, gene therapy will only prove to be an effective long-term treatment if the 'corrected' cells are able to survive for a long time or transmit the correction to daughter cells during mitotic cell division. Finally, effective gene therapy requires sufficiently high expression levels of the gene product in the target tissue. This depends on both the efficiency of expression from a single cell and also the total number of cells within the target tissue expressing the gene product. The former is controlled by factors which regulate the efficiency of gene expression; the latter depends upon the proportion of cells which have been successfully corrected. Two basic methods of introducing genes into the genomes of target cells are used: retroviral infection and gene targeting by homologous recombination. Although retroviral infection is undoubtedly superior as regards efficiency, homologous recombination may ultimately prove to be the method of choice by virtue of its ability specifically to 'target' and then replace, rather than complement, the defective gene.
At the present time, taking these criteria into consideration, adenosine deaminase deficiency, Lesch-Nyhan syndrome, phenylketonuria, α/β-thalassaemia, Gaucher's disease and α1-antitrypsin deficiency can be seen as examples of those genetic diseases most amenable to being 'cured' by gene therapy.

Retroviral vectors

The next three lectures concentrated upon the use of retroviral vectors as agents for the efficient introduction of DNA into mammalian cells — a critical requirement for successful gene therapy.

Dr R. Mulligan (Massachusetts Institute of Technology) compared and contrasted two potential target tissues for gene therapy — bone marrow and liver — noting that the former, although easily accessible, was resistant to the efficient introduction of DNA, whilst the opposite was true for liver hepatocytes.

He then discussed the advantages of using retroviral vectors for gene therapy, noting the highly efficient manner in which these viruses can infect cells and then become incorporated into their genome. This overcomes the problem of certain cell types being refractory to transfection and efficient integration of the introduced DNA into the host genome — two of the major obstacles to be overcome before gene therapy by homologous recombination can be considered. In addition, the retroviral vector approach benefits from the high proportion of cells which survive the procedure and can then be successfully reimplanted into the patient.

The 'cut and paste' protocol used for integrating DNA into cellular genomes with the use of retroviral vectors consists essentially of replacing the 'gag', 'pol' and 'env' genes of the wild-type retrovirus with the gene of interest. The recombinant retroviruses, lacking the essential protein products from these three genes, are unable to infect cells. Stocks of infectious recombinant retrovirus particles containing these three essential proteins are produced by transfecting the recombinant retrovirus into the cells of a 'helper' line whose genome contains the integrated pro-viral form of a packaging-deficient retrovirus. The provirus is able to provide the 'gag' and 'env' proteins necessary for the packaging of the recombinant retrovirus but cannot itself be packaged since its genome lacks the ψ- packaging signal. After retransplantation of the infected cells into the host tissue, the recombinant retroviruses remain dormant in the genome, unable to infect other cells in the absence of the 'gag', 'pol' and 'env' gene protein products. Hence, there is no danger of rampant retroviral infection spreading to other cells in the body.

Severe combined immunodeficiency (SCID) caused by adenosine deaminase deficiency is a good candidate for gene therapy since replacement of this single gene locus, expressed in all tissues and therefore requiring no cell-specific regulation, should cure the condition. Promising experiments involving the in vitro culture of murine bone marrow cells, infection with recombinant retroviral vector constructs containing the adenosine deaminase gene and subsequent retransplantation into lethally irradiated mice were reported to restore immune function. Although the mouse has given important information about basic requirements, before gene therapy is attempted in patients future experiments are planned using a monkey model which more closely resembles the human situation.

Certain forms of familial hypercholesterolaemia result from a deficiency of the cell receptor for low density lipoprotein (LDL). A rabbit strain — the Watanabe rabbit — deficient in the LDL receptor serves as an excellent experimental model to investigate the feasibility of using gene therapy to cure this condition. Recombinant retroviral vectors have been developed and then used to infect endothelial cells of the intrahepatic blood vessels. The sites of expression of the introduced gene were visualised with fluorescence microscopy techniques. Replantation of the infected cells resulted in a lowering of plasma cholesterol levels for 3–7 days, but elevated cholesterol levels had returned after 14 days. In the ensuing discussion, the development of antibodies to the LDL receptor was proposed as a possible explanation for the lack of continuing effectiveness.

A critical requirement for successful gene therapy is the ability to transduce and retransplant a sufficiently high number of cells expressing the introduced gene. To this end, experiments were reported utilising 'synthetic' arteries produced by retroviral infection of endothelial cells with β-galactosidase, followed by in vitro expansion of the cell population and then grafting onto canine carotid artery. This resulted in sustained and adequate expression levels of the enzyme.

Professor H. Green (Harvard Medical School) considered the potential of keratinocytes as target cells for gene therapy. This type of cell is an attractive proposition for gene therapy since its location in the epidermal layer of the skin renders it easily accessible. In addition, keratinocytes are easily cultured in vitro, another important consideration when assessing the suitability of a particular cell type for gene therapy. Indeed, a five-thousand-fold increase in the mass of keratinocytes can be achieved after just three weeks of in vitro cultivation. During in vitro cultivation the keratinocytes grow as a sheet of cells; consequently, the process of retransplantation is greatly simplified. The long-term survival of keratinocytes following retransplantation is graphically illustrated by the successful use of skin grafting to treat third degree burns patients. Analysis of these grafts several years post-operatively has demonstrated that the grafted skin is histologically more or less normal, although it lacks hair and sebaceous glands.

Recombinant retroviruses containing the gene coding for human growth hormone have been used to infect murine keratinocytes. In vitro cultures of these infected cells secrete active hormone into the growth medium. These cells were then grafted onto athymic mice (mice lacking a thymus gland). Despite demon-
stable local production of growth hormone from the grafted cells, none could be detected in the bloodstream. The reason for this is unclear but may reflect inefficient transport of growth hormone across the basement membrane separating epidermis and dermis. Subsequent experiments in mice using retroviral constructs containing a variety of different promoters to direct expression of the growth hormone gene have proved more successful. For instance, human growth hormone is still detectable in the circulation 28 days after retransplantation when the metallothionein promoter is used. Extrapolation to humans using this system, assuming equivalent levels of expression would require a 360 cm² skin graft, which Dr. Green thought was a feasible proposition.

Professor G. G. Brownlee FRS (Sir William Dunn School of Pathology, Oxford) discussed current research directed towards gene therapy for haemophilia B (Christmas disease) patients. This condition, the rarer of the two forms of haemophilia, is caused by a congenital deficiency of active plasma factor IX. Current symptomatic treatment, involving infusion of factor IX concentrate when required, is satisfactory but not ideal. A constant source of endogenously produced factor IX, provided by gene therapy, would represent a significant advance in treatment of the condition. Factor IX is normally expressed in a tissue-specific manner by liver hepatocytes. In common with a number of other proteins, factor IX undergoes a series of post-translational modifications prior to secretion from the hepatocytes into the bloodstream. These modifications include γ carboxylation of 12 glutamic acid residues at the amino terminus of the mature plasma protein, which is known to be essential for the expression of full biological activity. Carboxylation of these residues is catalysed by the vitamin K dependent carboxylase enzyme. This serves to illustrate the general point that for some proteins, such as factor IX, the concomitant expression of other enzymes will be required for successful gene therapy. The point was made that, provided the chosen target tissue produces sufficient levels of γ carboxylated factor IX which is then efficiently secreted into the bloodstream, there is no need to use the natural site of expression in the liver. Accordingly, skin was considered as a possible target tissue since retransplantation would be a relatively non-invasive surgical procedure. In the case of Christmas disease patients, this is of course an important consideration.

Retroviral infection of skin fibroblasts with vectors containing factor IX cDNA linked to various promoters has been shown to secrete low levels of fully active factor IX in vitro. Unfortunately, normal rat, mouse or human skin derived fibroblasts infected with retroviral vectors containing factor IX cDNA linked to various promoters secrete factor IX into the bloodstream only transiently and factor IX can no longer be detected 8 days after grafting. This combination of low expression levels and transient production in fibroblast experiments means that keratinocytes are now being considered as a possible alternative. Preliminary results suggest that retrovirally infected keratinocytes can express factor IX in vitro, albeit with only 20% of the natural biological activity.

Gene targeting

The next lecture considered the potential of homologous recombination of cells as an alternative means of correcting gene defects.

Dr K. Thomas (University of Utah, Salt Lake City, USA) explained how homologous recombination can specifically replace a defective gene with its wild-type counterpart. Unlike retrovirally mediated gene therapy, this targeted approach runs no risk of random integration into a part of the genome which may be critical for normal cellular function.

One long-term aim of this work is to ‘make’ mice with targeted mutations which would then act as animal models for a number of human diseases.

Mouse embryonic stem (ES) cells have been used as a model system. ES cells, with exogenous DNA introduced by microinjection techniques, were inserted into murine blastocysts prior to their implantation into foster mothers. The resulting chimaeric offspring demonstrated successful germ-line transmission of the targeted replacement of a mutant gene. Approximately 1 in 1,000 cells are estimated to contain the targeted correction. Since only 1 in 10 ES cells can be ‘infected’ by microinjection, the true figure for homologous recombination is nearer 1 in 100. Experimentation revealed that the DNA must be linear for efficient integration, but that neither the number of molecules injected nor the number of target sites within the host DNA affected the efficiency of integration. Electroporation was used as an alternative means to microinjection for introducing DNA into the cells. The gene coding for neomycin resistance was used in this test system. For efficient selection of homologous recombinants, the neomycin resistance gene construct (flanked with DNA homologous to hypoxanthine phosphoribosyltransferase) was designed such that homologous recombination would introduce the neomycin resistance gene into the hypoxanthine phosphoribosyltransferase (HPRT) locus, simultaneously excising the DNA sequence encoding exons 7 and 8 of HPRT. Hence, those wild-type neo⁺, HPRT⁺ cells, having successfully undergone homologous recombination, are easily detectable by their altered phenotype (neo⁻, HPRT⁻). Experimentation with the number of base pairs homologous to HPRT in the construct (4,000 or 9,000 bp) shows that, although electroporation is equally efficient in each case, the proportion of homologous recombinants is approximately four-fold greater for the constructs with 9,000 bp of homology (1 in 950 compared to 1 in 4,000). In summary, it is possible, with the use of neomycin resistance as a selectable marker, successfully to target DNA to the HPRT locus at a frequency of approximately 1 in 1,000.

*neo⁺ = neomycin sensitive; neo⁻ = neomycin resistant
HPRT⁺ = HPRT positive; HPRT⁻ = HPRT negative
(representing a 1,000-fold increase on the efficiency obtained using an analogous system with no selectable marker). The proportion of cells with the introduced DNA correctly integrated can be further enriched by selecting against incorrectly integrated neo' cells. Briefly, the targeting vector is constructed with the thymidine kinase (TK) gene downstream to the 3' region of homology. Homologous recombinants will not contain this sequence and will therefore be resistant to gancyclovir (GANC). Conversely, non-homologous recombinants, having integrated the TK gene, will be GANC' sensitive and can therefore be selected against on this basis. Applying this technique to the system described above results in 19 out of 24 surviving cells being homologous recombinants. In reality, it will not always be possible, as it is in this instance, to select for the 'target' gene product (hypoxanthine, aminopterin and thymidine [HAT] selection against HPRT' cells in this case). Nevertheless, data obtained from targeting the int-2 oncogene with selection for neo' (using G418) and GANC' looks promising with 4 of 81 surviving clones being homologous recombinants. One potential criticism of this system is the resultant expression of a foreign antigen (conferring neomycin resistance) in the engineered cells. A theoretical scheme (currently under investigation) to eliminate the neo' gene prior to retransplantation was outlined. In conclusion, it is likely that the adoption of the system described in this lecture could make gene therapy by homologous recombination feasible in many instances.

Regulation of gene expression

The final two lectures concentrated upon current research utilising transgenic methods which is shedding new light upon the regulation of gene expression.

Dr D. Melton (University of Edinburgh) described a transgenic mouse model for Lesch–Nyhan syndrome (HPRT deficiency). Although the mouse model does not spontaneously show the neurological disorders characteristic of the human condition, some symptoms can be induced by the administration of amphetamines which are thought further to decrease the already depleted levels of dopamine in the brains of these mice. The transgenic mice are unable to produce HPRT due to the absence of 10 kilobases (kb) from the 5' end of the HPRT gene including the promoter and first two exons of the HPRT gene. Homologous recombination was used in an attempt to complement the defective gene using constructs comprising the first two exons together with either 650 bp fragment comprising the normal promoter, a 'core' promoter sequence element or the metallothionein promoter. In the absence of any selection procedures it was reported that homologous recombinants were present in each case at a proportion of approximately 5 in 10 cells. However, of the cells which had any integrated exogenous DNA at all, approximately 1 in 100 were homologous recombinants. An encouraging finding for gene therapy was that Southern blot analysis of DNA from these cells showed that homologous and non-homologous recombinational events never occurred simultaneously in the same cell.

Transcription levels of HPRT in different tissues for each of the three transgenic lines were assayed by 'northern' blotting and compared to wild-type values. In no cases were expression levels equivalent to the wild-type control. However, relative expression levels in different tissues, with the exception of the lungs, showed a similar pattern to wild-type in both the 650 bp natural promoter and 'core' promoter transgenic lines. Two possible alternatives could explain the low levels of expression relative to wild-type. Either the 650 bp or 'core' promoter sequences do not replace all the control elements present in the 10 kb of missing sequence, or the plasmid sequences introduced into the gene as a consequence of homologous recombination downregulate transcription. The second of these possibilities was tested by constructing the targeting vectors so that homologous recombinants no longer contained any integrated plasmid sequences.

An elegant series of experiments demonstrating the importance of regulatory elements several kb away from the classical promoter region, which lies immediately 5' to the transcriptional start site, was described by Dr F. Grosveld (National Institute for Medical Research, London). The implications for gene therapy are obvious; it can no longer be assumed that introducing the structural gene and classical promoter in isolation will result in maximal expression. This provides another strong argument in favour of using homologous recombination rather than retroviruses for gene therapy since the former will target the gene to its natural position in the genome and hence put it under the control of any such distant regulatory elements.

A Dutch patient presenting with β-thalassaemia has been shown to have a deletion which includes a region extending 15 kb upstream from the adult β-globin gene. Detailed analysis of this region revealed the presence of a position independent sequence—the 'dominant control region' (DCR)—which enhances transcriptional activity in the β-globin gene cluster 10 kb downstream. A similar sequence has been found in the 5' region of the CD2 gene. Linking the β-globin structural gene to this CD2 control sequence has allowed the expression of this normally erythroid-specific gene product in T-cells.

The DCR 15 kb which is known to contain four DNAase I hypersensitive sites — regions of 'naked' DNA likely to be involved with transcriptional regulation—was then studied in further detail. Transgenic lines containing different sequence elements from within this region were constructed. Analysis of these constructs revealed that the DCR is a composite of
three approximately 250 bp sequence motifs centred around three of the four DNAase I hypersensitive sites. For efficient tissue-specific expression the DCR together with a 100 bp region containing the classical TATA and CAC 'boxes' are required. In addition, a sequence motif located in the second intron of the adult β-globin gene is required for correct mRNA processing. This latter point should be borne in mind when considering the use of intronless cDNA constructs for gene therapy. This in-depth understanding of the regulatory mechanisms controlling globin gene expression has allowed the recent development of a transgenic mouse model for the sickle-cell trait which will increase our understanding of this common inherited disease.

In summary, the day’s lectures demonstrated that rapid progress made in recent years now makes the ‘cure’ of genetic disease by gene therapy a realistic proposition.

The conference on Gene Therapy was held at the Royal College of Physicians on 1st March 1990.