Gene Transfer Moves Ahead

Investigators can now transfer practically any isolatable gene into cultured cells; their main goal is to study gene control

The possibility of genetic engineering—the transfer of new genes and thus new information into the cells of living creatures—can raise visions of either medical miracles or brave new worlds, depending on one's point of view. As a recent example, an attempt to perform gene therapy on two human patients attracted intense public interest as well as its share of "friendly fire" (*Science*, 31 October, p. 509).

Although many investigators thought the experiment premature, gene transfer research has made considerable progress in the past few years. Most of it has been done with cultured cells, however. Investigators have just begun to achieve some success in introducing new genes into laboratory animals.

What sets the recent work apart from earlier gene transfer experiments is the use of pure preparations of individual genes, products tailored by recombinant DNA technology to fit the researcher's needs. Formerly, gene transfer was achieved by techniques such as cell fusion or chromosome uptake, in which the investigator had little control over what genes were transferred.

However the transfer is achieved, the problem is to pick out the few cells that acquire the new gene from the many that do not. Usually investigators have used a gene that will confer some kind of selective advantage on the recipient cells and permit them to grow under conditions in which cells without the gene cannot grow. Earlier work was also limited by this requirement for transferring only selectable genes, or those closely linked to a selectable gene on the donor cell chromosomes. Now methods are being developed for putting any gene into cells.

Three of the major methods currently under investigation are incubation of the intended recipient cells with DNA precipitated with calcium phosphate, direct injection of DNA into the cellular nucleus, and use of vectors, usually modified viruses, that can carry genes into cells. Frank Graham and Alex van der Eb of McMaster University in Hamilton, Ontario, originally observed in 1973 that precipitation of DNA with calcium phosphate greatly enhanced gene uptake by cells. Then, in about 1977, Richard Axel, Saul Silverstein, and Michael Wigler of the College of Physicians and Surgeons of Columbia University began to use the method to transfer genes, including a viral gene and a number of cellular genes.

Their goal, like that of most researchers investigating gene transfer, is to study the control of gene expression in mammalian cells, one of the more recalcitrant mysteries of molecular biology. Axel says, "What modulates the level of expression of a gene is not obvious from looking at its primary structure." But with recombinant DNA technology it is now possible to modify gene structure in a systematic fashion before transfer to see how the changes affect transcription of the gene into messenger RNA, subsequent messenger processing, and ultimate translation of the message into protein structure. These questions are just beginning to be answered, as investigators have mainly concentrated on developing transfer methods and understanding how they work.

Axel, Silverstein, and Wigler first transferred a gene which they isolated from herpes simplex virus and which codes for the enzyme thymidine kinase (TK). The recipients were mutant cells deficient in the enzyme. Such cells cannot grow in a particular culture medium (called HAT because it contains hypoxanthine, aminopterin, and thymidine), but cells that are transformed by the tk gene and acquire the ability to make the enzyme can. (Transformed here means that the cells have acquired a new gene. Cells that have underdone malignant changes may also be called transformed. but this is a different matter.) The Columbia investigators, as well as others, went on to show that the calcium phosphate method could be used to transfer a number of additional genes for which selection methods are available.

Studies of bacterial transformation have shown that only a small portion of the population is amenable to transformation, but that bacterial cells which acquire one gene are likely to acquire others, too. Taking their cue from this work, Axel, Silverstein, and Wigler set out to see whether such cotransformation might also occur in mammalian cells. They found that it does. Wigler says, "Cells that take up the selectable gene may also take up unselectable genes." As many as 80 percent of mammalian cells transformed by the tk gene acquired the nonselectable gene, one coding for a modified human growth hormone. The overall efficiency of transformation by the calcium phosphate method is low, however. Only about 1 in 10⁵ or 10⁶ cells acquires the selectable gene when incubated with 50 picograms of cloned gene. The efficiency can be increased by using more DNA, however.

The selectable and nonselectable genes do not have to be linked before incubation with the recipient cells for cotransformation to occur, although this can be done. In one such case, Charles Weissmann and his colleagues at the University of Zurich used the calcium phosphate method to transfer a rabbit gene for β -globin, which they had joined to a *tk* gene, into mouse cells. But even if the genes are separate, they may become tied together after entering the cell and the ligated genes may then be integrated into a chromosome.

Several lines of evidence support the theory that transforming genes are integrated into chromosomes. For example, experiments from the laboratory of Frank Ruddle at Yale University showed that some transformed cell lines are unstable in that they progressively lose the foreign gene. Others are stable and retain it even in the absence of a selective pressure. With Kenneth Huttner and George Scangos, Ruddle showed that in stable lines the presence of an acquired *tk* gene was associated with specific chromosome, although with a different chromosome in each of the different lines.

Even more direct evidence for chromosomal integration comes from the Axel group, which used a radioactively labeled DNA probe to locate a transferred gene in each of four cell lines. They, too, found that the gene could be traced to a particular chromosome, but again it was different in different lines.

Evidence suggesting that separate pieces of transforming DNA become closely linked on the recipient chromosome was obtained by Wigler, who is now at the Cold Spring Harbor Laboratory. With Manuel Perucho and Douglas Hanahan, he cotransformed cells with the tk gene plus a nonselectable gene and showed that when the tk gene is lost the other gene disappears with it. Not only are the two genes lost at the same time, they can also be transferred together to new recipient cells.

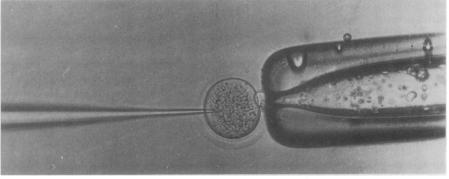
That the genes are closely linked does not necessarily mean that they were joined together before chromosomal integration, but the Cold Spring Harbor workers have evidence that this is the case. They can show that two added DNA's are consistently adjacent to one another on the chromosome, which suggests that they were joined before integration. If they had merely entered nearby sites, they would ordinarily be flanked not by each other but by cellular DNA, and this was not what was found.

In addition, the Ruddle group observed that the tk gene becomes part of a large DNA molecule, even in unstable lines, where they can find no evidence for chromosomal integration. All in all, the results support the idea that the DNA's taken up by cells do become linked before they are incorporated into a chromosome. The unstable lines lose the joined DNA's before they integrate.

These results mean that cotransformation with a selectable gene provides a way of introducing virtually any gene into cultured cells. There is a hitch, however. Jon Gordon, another of Ruddle's colleagues at Yale, cautions, "The cell is not under duress to keep the nonselectable gene." The selectable gene must be maintained and its protein product made if the cells are to survive under the selection conditions. But even if the other gene is kept, there is no guarantee that its product will be made. Moreover, the Ruddle group finds that added DNA's may be broken and rearranged before they are linked, another factor that may reduce the likelihood of getting a working nonselectable gene into cells.

Although the efficiency of transformation by calcium phosphate precipitates of DNA is low, that by direct injection of DNA into the nucleus may be much higher. When Mario Capecchi of the University of Utah injected the nuclei of cultured cells with a bacterial plasmid bearing the tk gene, 50 to 100 percent of the cells showed TK enzyme activity. A much smaller proportion of the injected cells—about 1 in 500 or 1000—were able to grow in HAT medium, however. Why so many cells, which appeared to have acquired TK activity, failed to survive in HAT medium is unclear.

Because of the greater efficiency of the injection technique, Capecchi has suggested that the method might be a way of



Injecting DNA into the pronucleus of a fertilized mouse egg

Although the tip of the micropipette has a diameter of only about 0.5 micrometers, the injection, Gordon says, ''is equivalent to your being speared by a telephone pole.'' Still, about 75 percent of the eggs survive the treatment. [Source: Jon Gordon, Yale University]

introducing new genes into embryos. This would be impossible by the low-efficiency calcium phosphate method because not enough embryos could be prepared.

In fact, Ruddle and Gordon injected the nuclei of fertilized mouse eggs with foreign genes and obtained evidence that the genes are retained in some of the animals that develop. "We are interested," Ruddle says, "in having the material get into the germ line; we want it passed on to a new generation." In this way, it would be possible to study the expression of a foreign gene, not just in cultured cells but in living animals.

Of the 180 mice that have so far developed from injected eggs, three proved to carry the foreign DNA, which consisted of a recombinant molecule originally containing a viral tk gene plus segments of bacterial plasmid and SV40 DNA's. All three mice had sufficient quantities of the foreign DNA to suggest that most or all of the mouse cells contained the DNA segments, indicating that the DNA must have replicated.

In one animal, the foreign segments, which consisted primarily of plasmid DNA, were not integrated into the chromosomes. This could mean that the plasmid replicated independently or that the segments were copies of a sequence that was integrated. In the other two animals, however, the foreign DNA segments were integrated. Both included material from each of the three DNA's of the original recombinant molecule. One seemed to have an intact TK sequence. All the animals were killed at birth, but eventually Ruddle and Gordon hope to produce breeding animals bearing a foreign gene in their germ cells. "But first we have to learn how to get the genes in at a higher frequency and maintain their integrity," Ruddle explains.

Beatrice Mintz of the Institute for Cancer Research has been working for some time to develop methods for introducing foreign genes into living animals. She maintains, "I don't think we will get definitive answers about mammalian gene expression just from tissue culture work."

One system she has developed uses teratocarcinoma cells—cancer cells with the capacity to differentiate into many different cell types. When they are injected into early mouse embryos, the mice that develop are mosaics of cells derived from both normal embryonal and teratocarcinoma cells—which in the embryonic environment lose their malignant properties and develop normally.

With the new gene transfer techniques being developed, Mintz thinks it will be possible to introduce foreign genes into cultured teratocarcinoma cells before they are injected into the embryos. She and Axel have shown that such cells take up the tk gene and that it is expressed and kept even in the absence of a selective pressure. Cells transformed in this manner maintained the gene when they were injected into mice and formed tumors.

The principal problem holding up the intended experiments with embryos is the fact that teratocarcinoma cells that are maintained in culture have abnormal numbers of chromosomes. The results of experiments with them might not reflect normal gene expression. Earlier embryo studies used teratocarcinoma cells that were maintained as transplantable tumors and were chromosomally normal. Mintz thinks that she may have identified a cultured teratocarcinoma cell line that does have a normal chromosome composition, but further work will be needed to confirm this.

A different approach to the introduction of foreign genes into living animals has been taken by Martin Cline, Karen Mercola, Winston Salser, and their colleagues at the University of California at Los Angeles (UCLA). They withdraw bone marrow cells, incubate them with calcium phosphate precipitates of the desired gene or genes, and inject the cells back into the animal. With this approach they were able to transfer two different genes into the bone marrow of mice (*Sci*ence, 25 April, p. 386).

The UCLA team is the one responsible for the gene therapy attempt on the two human patients, who suffer from a severe, usually fatal, form of anemia caused by the inability to make β -globin. In July of this year, the researchers applied their transfer method in the hopes of putting a human gene for the protein into the patients' bone marrow cells. At first there were no indications that the Cancer Institute, developed a new generation of virus vectors that allow the cell to survive, although the efficiency of transformation is greatly reduced.

The new vectors are ingeniously constructed recombinants consisting of bacterial plasmid DNA that permits multiplication of the vectors in bacteria and thus production of large quantities of the material for transfer experiments, SV40 DNA that is missing the gene coding for the major coat protein, and the gene or genes to be transferred. Because the SV40 DNA cannot direct the synthesis of any viral proteins, complete viral particles cannot be made and the recipient

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transfers worked. But Cline now says that they are beginning to see "encouraging" results, which he declined to specify on the grounds that the data are not yet ready for publication. If the encouragement turns out to be justified, this could be a step on the road to those medical miracles.

Paul Berg of Stanford University has been interested for many years in the idea of putting genes into mammalian cells and has primarily emphasized the development of viral vectors. "It was," he points out, "our motivation for trying to construct recombinant DNA molecules." In particular, the Berg group has worked with the SV40 virus and has shown that genes introduced into an appropriate region of the viral DNA will be expressed in cells transformed by the recombinant molecule. For example, a DNA segment coding for rabbit β -globin was put into monkey cells in this way, "The cells produced large quantities of the β -globin, as much as they would have produced if the sequence had coded for the SV40 coat protein." In a similar manner, Dean Hamer and Philip Leder of the National Institute of Child Health and Human Development used an SV40 vector to transfer a mouse gene for β globin into monkey cells, which acquired the ability to make the mouse protein.

The vector designs used for these experiments have the advantage of permitting very efficient gene transfer. Unfortunately, virus replication eventually kills the cells and their long-term study is not possible. More recently, Berg, Richard Mulligan, also of Stanford, and Bruce Howard, who is now at the National cells are not killed. But the viral DNA does contain control regions for expression of the transferred gene.

Using these vectors, Berg and Mulligan transformed mammalian cells with a gene from the bacterium *Escherichia coli* that codes for the enzyme xanthineguanine phosphoribosyltransferase. The bacterial enzyme is made in the recipient cells and corrects the enzyme deficiency of cells taken from a patient with Lesch-Nyhan disease. This condition, which usually kills its victims at an early age, is caused by the lack of the human equivalent of the *E. coli* enzyme.

The Stanford group has also worked out a procedure for selecting normal mammalian cells that have been transformed by the bacterial enzyme. It depends on the ability of the *E. coli* enzyme, but not of the mammalian enzyme, to use a particular substrate. Because the vectors developed by the Stanford groups can accommodate other genes in addition to the selectable one, this again means that there is a way of introducing nonselectable genes into cells—and the cells need not be mutants.

Even though most of the investigators doing gene transfer work emphasize the use of the methods for studying gene control and other fundamental problems in molecular biology, the implication that the methods might one day be used for genetic engineering is inescapable. Berg immediately throws cold water on the idea that his group has come up with a therapy for Lesch-Nyhan disease, however. He points out that all the cells of these patients lack the enzyme and it would be a formidable task to introduce the gene into all of them. In any event, there are questions about whether vectors derived from an animal tumor virus, such as SV40, should be used for genetic engineering in humans.

But the fundamental objection raised by many of the investigators concerns the paucity of current knowledge of gene control. Solving that problem is, after all, a major thrust of their research.

Says Axel, "I know of no one who has been able to insert a tissue-specific gene into an appropriate cell line and have its expression regulated in the normal way." Several investigators have shown, for example, that a transferred β globin gene will be expressed, but for the most part the recipient cells have been fibroblasts or some other cell type that does not ordinarily make the protein. When Axel, with Tom Maniatis of the California Institute of Technology, put the gene into mouse erythroid cells, which do make hemoglobin, they found that β -globin gene was copied into RNA but that the RNA synthesis was not regulated the way it should have been.

As Berg sums up the concern about the current situation, "There has been a lot of loose talk about gene therapy, but people don't understand the primitive state of the knowledge." Researchers fear that if the gene is improperly controlled it may not help, and might even hurt, the recipient.

Many investigators think that it may be necessary to put a transferred gene into its natural chromosomal site to get normal control. With one exception, there is now no way to put a gene in a particular chromosomal location. The exception is a method devised by Ronald Davis and Stewart Scherer of Stanford University for substituting an altered gene for its normal counterpart. The catch is that so far the method is applicable only to yeast.

Presumably, if the DNA segments that control expression of a given gene were identified, it would be possible to transfer them along with the gene. If this DNA complex were to insert in the wrong place, however, it might disrupt the control of other genes.

Finally, there is the observation by Ruddle and his colleagues that transferred DNA may be scrambled before integration. They and other investigators have also noted that the chromosomes tend to break easily at the site of gene integration. Whether this tendency preceded integration or resulted from it is unknown. Says Ruddle of the possibility of gene therapy, "I think it is in the cards, but we still have to get these points under control."—JEAN L. MARX