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GENE THERAPY

Synthetic chromosomes are real
Innovative DNA manufacture
Making gene therapy safer

CONTRACT MANUFACTURING

Buying process production

PROCESS PLANT

Cooking up corporate success

***DAVID THATCHER, ROCKY CRANENBURGH** and **JULIAN HANAK** of **COBRA THERAPEUTICS LTD**,* *explain their approach to antibiotic and ribonuclease-free manufacture of low endotoxin plasmid DNA for gene therapy. In a nutshell, using host-cell genetic engineering to improve the quality and safety of biological products.*

INNOVATION IN DNA MANUFACTURE

Genetic engineering has revolutionised the manufacture of biological products. The technology has provided access to novel pharmaceutical agents and enabled a marked advance in the quality and safety of biological products. The single most important factor in this improvement has been the introduction of novel gene control systems to increase the cellular productivity of the source (host) material.

However, quality has also been improved by the ability to select host systems with lower intrinsic risk than traditional sources of biological products; blood, animal tissues and attenuated micro-organisms. Host cell systems for the production of new biological products can now be selected that have no pathogenic traits or known adventitious contamination and have a history of safe use (eg *E.coli* K12 strains, Chinese hamster ovary cells). Nevertheless, it is apparent that these new production processes still carry a number of real risk factors, as anyone seeking regulatory approval for clinical trial of a new product knows only too well.

Quality concerns usually focus on validating the removal of incipient or excipient hazardous compounds from the product, or minimising, removing or inactivating potential or real adventitious agents present in the raw materials. Most processes use either biological ingredients or ingredients of biological origin that can carry additional risk factors (eg viral infection or new-variant CJD from animal-derived products) or hazardous chemical components which can cause toxaemia or allergic reaction. Although modern high-resolution separation technologies are expected to be able to control such hazardous impurities, modern approaches to quality assurance dictate that, where possible, the risk should be eliminated. Random selection can often be used to reduce risk factors, for example adapting cell lines to animal-product-free media so that components potentially contaminated with infectious agents need not be used; in other cases, risks can only be avoided or minimised through rational manipulation of the host cell genome.

MANUFACTURE OF PLASMID DNA

Although isolation at the sub-milligram level is a routine, kit-based laboratory procedure, gram scale manufacture for therapeutic use is a highly specialised activity,.

Most of the methods currently used in GMP manufacture of plasmid DNA for human gene therapy use *E.coli* K12-derived strains as the host cell and involve the steps described in the flow sheet shown in *Figure 1*. The stages involve the use of a

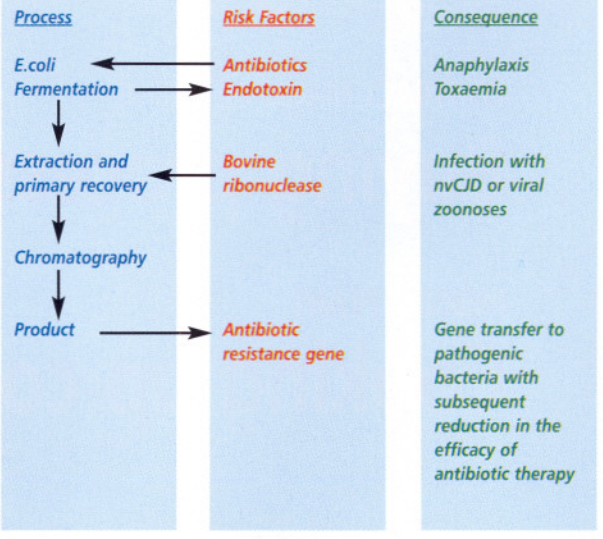
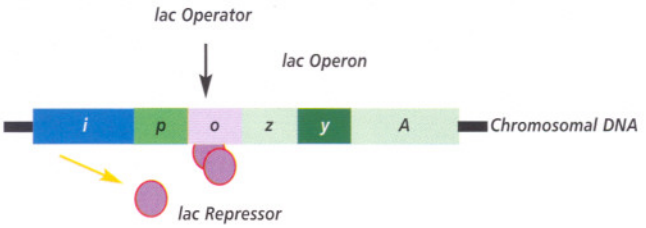


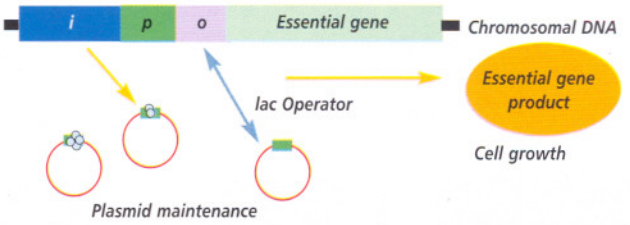
Figure 1 Flowchart describing plasmid DNA production

Figure 2a
Structure of conventional host strain



Plasmids are maintained by antibiotic selection. The E.coli chromosome contains a gene cluster (the lac operon) that is tightly regulated. The product of the I gene is a repressor that, in the absence of lactose, binds tightly to a 21bp sequence: the lac operator. When the repressor is bound to the operator, no gene expression can occur.

Figure 2b
Structure of ORT Host Strain



If the 21bp lac operator sequence is present on a plasmid, these operator sites will also bind repressor and thereby reduce (titrate) the level of free repressor in the cell. If the chromosomal lac operon is replaced by an essential gene under the control of a lac operator, then the cell becomes dependent on operator repressor titration to survive. If the level of plasmid drops, more repressor molecules are available for binding to the chromosomal lac operator of the new operon and essential gene synthesis is shut off. The plasmid free cells' ability to grow is thereby diminished.

number of raw materials that have inherent risk factors that could threaten patient safety if not controlled: endotoxin, antibiotics and animal products.

ENDOTOXIN

E.coli strains employed in plasmid production are selected on the basis of the absence of pathogenic determinants often associated with this species. Currently the only major risk factor directly associated with the K12 parental strains used is the presence of endotoxin, a natural component of the cell wall and therefore an incipient hazard present in the biomass feedstock. Endotoxin levels are easily measured and although often difficult to separate from plasmid DNA, there are robust methods available which will reduce levels to those acceptable for human use. In order to reduce the challenge to the separation process, strains can be selected with lowered endotoxin production or by knocking out genes involved with endotoxin synthesis such as the msbB gene.

ANTIBIOTICS

Plasmids used in gene therapy and all plasmids used to manufacture *E.coli*-derived recombinant protein products cannot be maintained without antibiotic selection. This intervention is necessary because segregation of these high-copy number plasmids within the dividing bacterial cell is uncontrolled.

In a growing culture, unless selected against, plasmid-free host cells will rapidly outgrow the plasmid-containing cells; a phenomenon known as segregational loss. Segregational loss during production of all plasmids currently used in the gene therapy can be prevented by incorporation of the *kan* or kanamycin resistance gene that codes for kanamycin phosphotransferase. Cultures are grown in the presence of the kanamycin and cells which lose the plasmid are killed by the antibiotic.

Although the use of ß-lactam antibiotics has been prohibited for the production of biological products for 20 years, the consequences of using other antibiotics and antibiotic resistance genes in gene therapy are only just being addressed.

Although kanamycin is not in wide clinical use in the West, the kanamycin-based antibiotic amikacin is an antibiotic of significance and is often used to treat life threatening septicaemia, being effective against methicillin-resistant *Staphylococcus*. Resistance to amikacin can be conferred by a single mutation in the kanamycin phosphotransferase gene. Options for removal of the *kan* gene in gene therapy products are therefore desirable, particularly in those indications involving widespread use (eg in DNA vaccines) or in cases where the patient is already at risk from sepsis (eg wound healing or cystic fibrosis).

Cobra Therapeutics has developed a simple system, which allows the design of a robust manufacturing process without the use of antibiotics at any stage of the process and without the use of any selectable marker on the plasmid. How can this be achieved? The answer is to move the structural gene involved in selection from the plasmid to the chromosome of the host strain and then make the regulation of that gene dependant on non-coding regulatory sequences borne on the plasmid. The mechanism is based on a phenomenon known as repressor titration. For example, if normal *E.coli* is transformed with a pUC-based plasmid, the *lac* operator sites on the plasmid interact with the *lac* repressor molecules within the cell causing derepression of ß-galactosidase transcription and

EMEA/CPMP Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products (1999)

'Selection markers, used during screening and development and remaining in the final product, should be carefully evaluated in the light of their potential to adversely impact on standard therapies for certain human diseases, for example, antibiotic resistance genes. Consideration should be given to avoid their use, where feasible.'

FDA Guidance for Industry. Guidance for Human Somatic Cell Therapy and Gene Therapy (1998)

'If antibiotic selection is used during production, it is preferable not to use selection markers which confer resistance to antibiotics in significant clinical use, in order to avoid unnecessary risk of spread of antibiotic resistance traits to environmental microbes. . . non antibiotic selection systems can also be used.'

synthesis of the enzyme. However, if the ß-galactosidase gene is replaced by a gene essential for growth, the cell then becomes dependant on the presence of plasmid and stable maintenance of the plasmid ensues. This system is called the Operator Repressor Titration or ORT system and is shown in

Figure 3
Antibiotics free plasmid maintenance by repressor titration

(a) *(b)* *(c)*

Plasmid stability visualised through gel electrophoresis of isolated plasmid after 0, 15, 55, and 72 generations. The plasmid is a pUC based plasmid containing the ampicillin resistance gene. Panel (a) shows stable maintenance of the plasmid grown under ampicillin selection. Panel (b) shows stable maintenance by repressor titration as the same strain is grown in the absence of antibiotic, but under conditions where derepression of an essential chromosomal gene is required for growth (in this case ß-galactosidase). Panel (c) shows segregational loss of the same plasmid when the strain is grown under conditions where repressor titration is not required for cell growth (growth using a glucose carbon source).

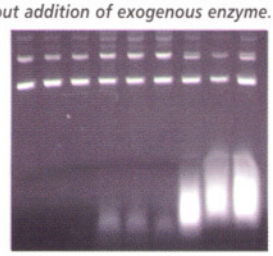
Figure 2b. A comparison of plasmid stability between an ORT host vector system and an antibiotic-selected plasmid grown in the absence of antibiotic is shown in *Figure 3*.

ANIMAL PRODUCTS

Animal products used in plasmid manufacture are bovine hydrolysates, used as media components, and ribonuclease, used to reduce RNA levels in the feedstock. These animal proteins are derived from a species (cattle) that has been shown to harbour a disease (BSE) that can be transferred, and is fatal, to man (new-variant CJD). As this disease is little understood and there is neither an adequate diagnostic method nor effective treatment, use of any bovine ingredient in a medicine is a significant risk factor. Quality assurance at present is based on sourcing the raw material from those parts of the world where the disease is absent.

Bovine media components may be straightforwardly substituted by vegetable protein. Replacement of bovine ribonuclease, however, is not so easily achieved. Bovine ribonuclease is attractive because the enzyme activity is particularly resistant to denaturation by the harsh chemical environment required in the extraction of the plasmid (1% SDS at pH 12.5). RNA is produced by the host cell at much higher levels than plasmid DNA and, as RNA has similar physico-chemical properties to DNA, its removal is difficult to achieve during manufacture. RNA also carries a coding capacity and can be considered a critical contaminant to be removed from any gene therapy product.

Figure 4
Gel electrophoresis of plasmid preparations of a host strain expressing bovine ribonuclease A shows that endogenous levels of RNA may be reduced without addition of exogenous enzyme.



One solution obviously is to use a recombinant source of the enzyme. Unfortunately, bovine ribonuclease is difficult to express at high concentrations in micro-organisms as the enzyme is toxic to the cell. The low level expression from these strains makes recombinant bovine ribonuclease difficult and expensive to obtain in quantity.

ONE SOLUTION IS TO. . .

Our solution has been to express the ribonuclease in the host cell, in a form that is sequestered until processing is initiated. The production strain can be engineered such that bovine ribonuclease is expressed from a chromosomal gene and, by the use of an optimised leader sequence, synthesis directed to the periplasmic space. The periplasmic location prevents accumulation of toxic amounts of ribonuclease in the cytoplasm while still retaining the enzyme within the cell. On lysis with alkaline SDS, ribonuclease is released and, during the extraction/renaturation procedure the level of RNA present is significantly reduced (*Figure 4*).

Although the use of genetic engineering of the host cell to improve quality can be time consuming and be seen to delay product development, over the lifetime of a product such upfront investment can have significant savings on cost. For example, the validation and monitoring of antibiotic removal from the product and the processing equipment can be extremely expensive. The use of animal products is becoming more and more highly regulated with traceability and veterinary certification of the source animals now being required for licensed products. The effect of this regulation has naturally caused a dramatic increase in the price of such raw materials. The potential for host cell modification to reduce the risk of biotechnology medicines to the patient population is enormous and also makes sense economically.

REFERENCES

HORN, N.A., MEEK,J.A., BUDAHAZI,G., AND MARQUET, M. (1995) HUMAN GENE THERAPY 6, 565-573.

VARLEY, D.L., HITCHCOCK, A.G., WEISS, A.M.E., HORLER, W., COWELL,R., PEDDIE, L., SHARPE, G.S., THATCHER, D.R. AND HANAK, J.A.J. (1999) BIOSEPARATIONS 8, 209-217.

SOMERVILLE, J. E., CASSIANO, L., BAINBRIDGE, B., CUNNINGHAM, M. D. AND DARVEAU, R. P. (1995) J. CLIN. INVEST. 97: 359-365.

PERLIN, M.H. AND LERNER, S.A. (1979) ANTIMICROB. AGENTS CHEMOTHER. 16, 598-604.

IRANI M; OROSZ L; BUSBY S; TANIGUCHI T; ADHYA S (1983) PROC NATL ACAD SCI USA (UNITED STATES) 80, 4775-9.

WILLIAMS, S.G., CRANENBURGH, R.M., WEISS, A.M.E., WRIGHTON, C.J., SHERRATT, D.J. AND HANAK, J.A.J. (1998) NUCLEIC ACIDS RESEARCH 26, 2120-2124 AND US PATENT 5,972,708

TARRAGONA-FIOL, A., TAYLORSON, C.J., WARD, J.M. AND RABIN, B. (1992) GENE 118, 239-245.