# United States Patent [19]

**Axel et al.**

[11] **4,399,216**

[45] **Aug. 16, 1983**


[54] **PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS**


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[21] Appl. No.: **124,513**

[22] Filed: **Feb. 25, 1980**

[51] **Int. Cl.**[3] ..... **C12N 15/00**; C12N 5/00; C12P 21/00; C12Q 1/68; C12Q 1/02; C12Q 1/04

[52] **U.S. Cl.** ..... **435/6**; 435/172; 435/240; 435/317; 435/811; 435/948; 435/29; 435/34; 435/68

[58] **Field of Search** ..... 435/68, 172, 70, 240, 435/241, 948, 811, 6, 29, 34; 424/85, 177, 178, 180



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[57] **ABSTRACT**

The present invention relates to processes for inserting DNA into eucaryotic cells, particularly DNA which includes a gene or genes coding for desired proteinaceous materials for which no selective criteria exist. The insertion of such DNA molecules is accomplished by cotransforming eucaryotic cells with such DNA together with a second DNA which corresponds to a gene coding for a selectable marker.

The invention further relates to processes for inserting into eucaryotic cells a multiplicity of DNA molecules including genes coding for desired proteinaceous materials by cotransformation with the desired genes and with amplifiable genes for a dominant selectable marker in the presence of successively higher amounts of an inhibitor. Alternatively, the insertion of multiple copies of desired genes is accomplished by transformation using DNA molecules formed by ligating a DNA molecule including the desired gene to a DNA molecule which includes an amplifiable gene coding for a dominant selectable phenotype such as a gene associated with resistance to a drug in the presence of successively higher amounts of an agent such as a drug against which the gene confers resistance so that only those eucaryotic cells into which multiple copies of the amplifiable gene have been inserted survive.


**73 Claims, 2 Drawing Figures**

COTRANSFORMATION OF EUCARYOTIC CELLS

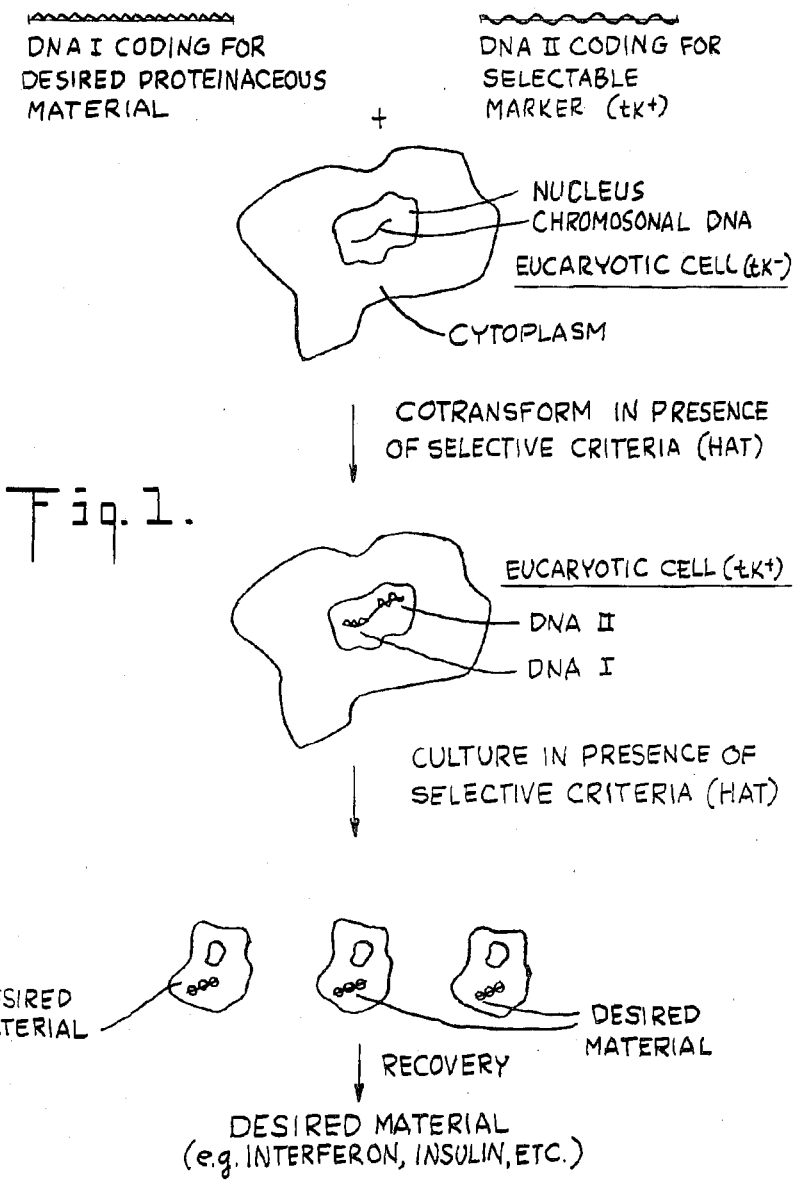


Fig. 1.

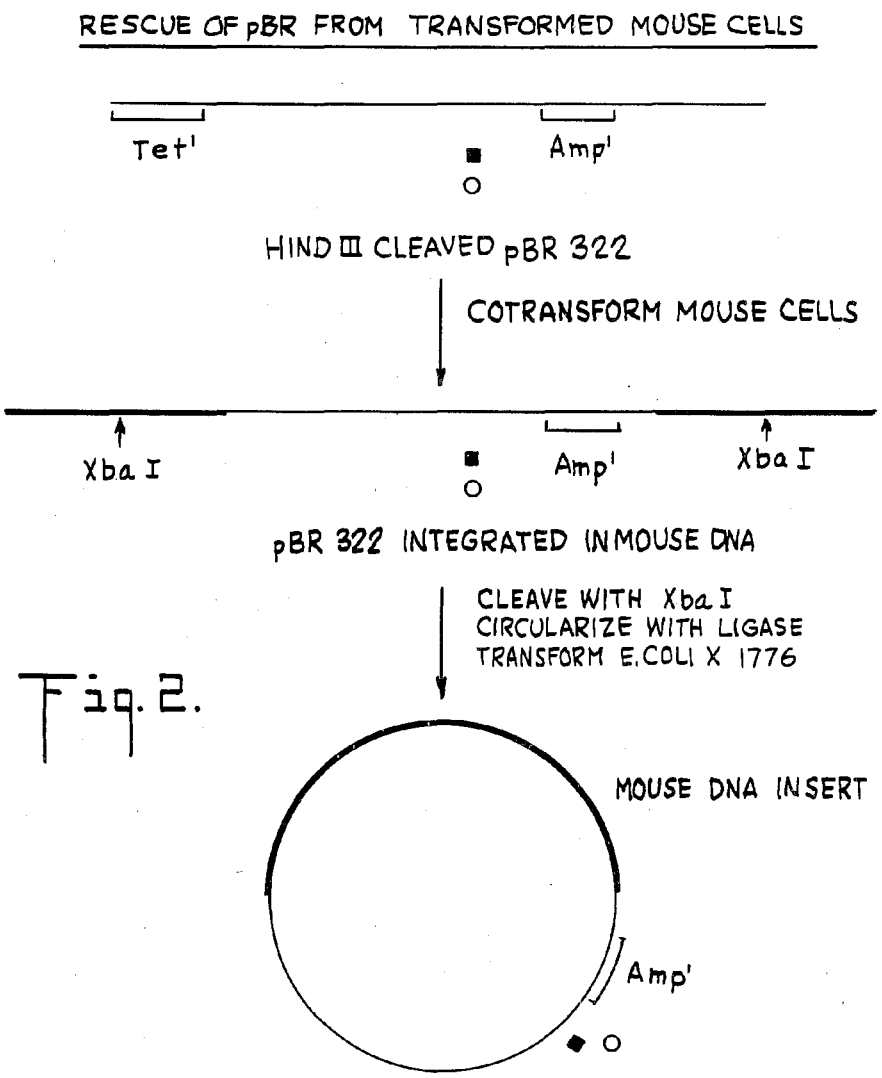


Fig. 2.

SCHEME FOR THE RESCUE OF BACTERIAL PLASMIDS FROM TRANSFORMED CULTURED CELLS USING DOUBLE SELECTION TECHNIQUES.

# PROCESSES FOR INSERTING DNA INTO EUCARYOTIC CELLS AND FOR PRODUCING PROTEINACEOUS MATERIALS

The invention described herein was made in the course of work under grants numbers CA-23767 and CA-76346 from the National Institutes of Health, Department of Health and Human Services.

## FIELD OF THE INVENTION

This invention concerns the introduction and expression of genetic informational material, i.e., DNA which includes genes coding for proteinaceous materials and/or genes regulating or otherwise influencing the production thereof, into eucaryotic cells, that is, cells of organisms classified under the Superkingdom Eucaryotes including organisms of the Plant and Animal Kingdoms. Such genetic intervention is commonly referred to as genetic engineering and in certain aspects involves the use of recombinant DNA technology. The invention disclosed is to be distinguished from the introduction of DNA into organisms of the Superkingdom Procaryotes including particularly bacteria. This distinction is based in part upon the basic differences between eucaryotic and procaryotic cells, the former being characterized by true nuclei formed by nuclear envelopes and by meiosis and the latter being characterized by the absence of well-defined nuclei and the absence of meiosis. Moreover, at the genetic level many genes in eucaryotes are split by non-coding sequences which are not continuously colinear, whereas in procaryotes, the genes are continuously colinear.

## BACKGROUND OF THE INVENTION

Although advances in the understanding of procaryotic organisms, particularly bacteria, having for the most part proceeded independently of advances in the understanding of eucaryotic organisms, it may be helpful to an appreciation of the present invention to set forth certain developments involving procaryotes.

In 1944, Avery reported the transformation of a procaryotic cell using DNA-mediated transfer of a cellular gene. Avery, O. T., et al., J. Exp. Med. 79: 137–158 (1944). Thereafter, reports of procaryotic transformation occurred in the literature. In 1975, Cohen and others reported results involving first transformation, then cotransformation of the procaryote *Escherichia coli*. Kretschmer, P. J., et al., J. Bacteriology 124: 225–231 (1975). In the experiments reported therein the authors disclosed the cotransformation of procaryotic cells using plasmid DNA, that is, extrachromosomal DNA which occurs naturally in many strains of Enterobacteriacae. In these experiments it was found that particular cells in a $CaCl_2$-treated bacterial population are preferentially competent for tranformation. However, the frequency of transformation and the stability of the transformants obtained was low, possibly because the plasmid is not incorporated into the chromosomal DNA. As a result, cotranformants lost acquired traits after several generations. In addition, these experiments with bacteria required the addition of a gene promoter to the transforming DNA in order to obtain expression.

Meanwhile, experiments with eucaryotic cells proceeded substantially independently of those with procaryotic cells. In 1962, Szybalska, E. H. and Szybalski, W. PNAS 48: 2026 (1962) reported the transformation of mammlian cells but with such low frequency of transformation that it was not possible to distinguish tranformants from cells which had merely undergone spontaneous reversion. Again, as with procaryotic cells, further reports of eucaryotic transformation occurred in the literature, but such results were oftentimes not reproducible by others. In addition, low frequencies of transformation, lack of understanding of the molecular basis for gene expression and the lack of molecular hybridization probes contributed to the lack of progress in this area. As a result, studies on the transformation of eucaryotic cells were essentially restricted to viral genes. Graham, F. L., et al., Cold Spring Harbor Symp. Quant. Biol. 39: 637–650 (1975) and McCutchen, J. H. and Pagano, J. S., Journal National Cancer Institute, 41: 351–357 (1968).

More recently, however, eucaryotic cells, specifically mammalian cells, were transformed with foreign DNA coding for a selectable phenotype. Wigler, M., et al., Cell 11: 223–232 (1977). This work has been extended and has resulted in the present invention wherein it has been discovered inter alia that eucaryotic cells can be cotransformed to yield transformants having foreign DNA integrated into the chromosomal DNA of the eucaryotic cell nucleus. Moreover, it has unexpectedly been discovered that such foreign DNA can be expressed by the cotransformants to generate functional proteins. In addition, by contrast with procaryotic transformants, the foreign DNA is stably expressed through hundreds of generations, a result that may be attributable to integration of the foreign DNA into the chromosomal DNA.

The present invention provides major advances over bacterial systems for future use in the commercial preparation of proteinaceous materials particlarly proteins of eucaryotic origin such as interferon protein, antibodies, insulin, and the like. Such advantages include the ability to use unaltered genes coding for precursors for such proteinaceous materials. After cellular synthesis, the precursor can be further processed or converted within the eucaryotic cell to produce the desired molecules of biological significance. This phenomenon is well known for insulin which is initially produced in the eucaryotic cell as preproinsulin which is then converted to active insulin within the cell by appropriate peptide cleavage. Since procaryotic cells lack the requisite cellular machinery for converting preproinsulin to insulin, the insertion into a procaryotic cell of the eucaryotic gene associated with insulin will result in the production of preproinsulin, not insulin. Although, in the case of insulin, a relatively small and well characterized protein, this difficulty can be overcome by chemical synthesis of the appropriate gene, such an approach is inherently limited by the level of understanding of the amino acid sequence of the desired protein. Thus, for interferon protein, clotting factors, antibodies and uncharacterized enzymes, for which the exact amino acid sequence is not yet known, a procaryotic system will likely not prove satisfactory. By contrast, a eucaryotic system is not associated with such disadvantages since the eucaryotic cell possesses the necessary processing machinery. It is thus one important object of the present invention to provide a process for producing desired proteinaceous materials such as interferon protein, insulin, antibodies and the like which does not require a detailed molecular understanding of amino acid sequence.

In addition to the problem of precursors having additional amino acids which must be removed to produce

active protein, important biological materials may be modified by chemical additions after synthesis and cleavage. Thus, for example, human-produced interferon is a glycoprotein containing sugar molecules in addition to protein. If produced in a bacterial cell, the interferon lacks the sugar molecules which are added when interferon is produced in a human cell. Moreover, proteinaceous materials produced within bacteria may include endotoxins which can cause inflammation if the proteinaceous material is administered to a mammal without significant purification. By contrast, interferon produced in a eucaryotic cell would be free of endotoxins.

It is therefore another important object of this invention to provide a process for producing compounds which include both non-proteinaceous and proteinaceous moieties such as glycoproteins which cannot be produced in bacterial cell.

## SUMMARY OF THE INVENTION

This invention provides a process for inserting foreign DNA into eucaryotic cells by cotransforming the cells with this foreign DNA and with unlinked DNA which codes for proteinaceous material associated with a selectable phenotype not otherwise expressed by the cell. The cotransformation is carried out in a suitable medium and in the presence of selective conditions permitting survival and/or identification of eucaryotic cells which have acquired the selectable phenotype. The process of this invention is particularly suited for the insertion into eucaryotic cells of DNA which codes for proteinaceous materials which are not associated with a selectable phenotype such as interferon protein, insulin, growth hormones clotting factors, viral antigens, antibodies and certain enzymes.

By use of the cotransformation process of the present invention is it possible to produce eucaryotic cells which synthesize desired proteinaceous and other materials and which can be grown in culture to produce these materials in quantities not obtainable with conventional technology.

In one embodiment of the invention, the cotransformation process can be used to insert multiple copies of genes coding for desired materials into eucaryotic cells. Alternatively, a multiplicity of foreign DNA molecules corresponding to multiple copies of a desired gene can be inserted into eucaryotic cells by transformation with molecules each of which is formed by linking a foreign DNA molecule to a second DNA molecule corresponding to an amplifiable gene for a dominant selectable phenotype not otherwise expressed by the cell. The transformation is then carried out in the presence of successively elevated concentrations of an agent permitting survival and/or identification of eucaryotic cells which have acquired multiple copies of the amplifiable gene. This approach is particularly useful when the dominant selectable phenotype is resistance to a drug which is lethal unless multiple copies of the drug resistant gene are present and the agent is the drug.

By inserting multiple copies of genes coding for desired materials into eucaryotic cells according to either of these approaches it is possible to produce eucaryotic cells which yield desired materials in high concentrations and which can be grown in culture to produce such materials in quantities not obtainable with conventional technology.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic flow diagram illustrating the cotransformation process in accordance with the present invention.

FIG. 2 is a schematic flow diagram illustrating a process for recovering foreign DNA I from cotransformed cultured cells using double selection techniques.

## DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

*Transformation* means the process for changing the genotype of a recipient cell mediated by the introduction of purified DNA. Transformation is typically detected by a stable and heritable change in the phenotype of the recipient cell that results from an alteration in either the biochemical or morphological properties of the recipent cell.

*Cotransformation* means the process for carrying out transformations of a recipient cell with more than one different gene. Cotransformation includes both simultaneous and sequential changes in the genotype of a recipient cell mediated by the introduction of DNA corresponding to either unlinked or linked genes.

*Proteinaceous* material means any biopolymer formed from amino acids.

*Genotype* means the genetic constitution of an organism as distinguished from its physical appearance.

*Phenotype* means the observable properties of an organism as produced by the genotype in conjunction with the environment.

*Selectable phenotype* is a phenotype which confers upon an organism ability to exist under conditions which kill off all organisms not possessing the phenotype. Examples include drug resistance or the ability to synthesize some molecule necessary to cell metabolism in a given growth medium. As used herein, selectable phenotypes also include identifiable phenotypes such as the production of materials which pass from or are secreted by the cell and can be detected as new phenotypes either by functional, immunologic or biochemical assays.

*Interferon protein* means the proteinaceous part of the glycoprotein interferon, that is, the portion remaining after removal of the sugar portion. It includes the protein portion of interferon derived from human leukocyte, fibroblast or lymphoblastoid cells.

*Chromosomal DNA* means the DNA normally associated with histone in the form of chromosomes residing in the nucleus of a eucaryotic cell.

*Transcription* means the formation of a RNA chain in accordance with the genetic information contained in the DNA.

*Translation* means the process whereby the genetic information in an mRNA molecule directs the order of specific amino acids during protein synthesis.

In accordance with the present invention, foreign DNA I can be inserted into any eucaryotic cell by cotransforming the cell with DNA I and with unlinked foreign DNA II which includes a gene coding for a selectable phenotype not expressed by the cell unless acquired by transformation. The cotransformation is carried out in a suitable growth medium and in the presence of selective conditions such that the only cells

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