1969

Transformation of mammalian cells by exogenous DNA

George E. Farley
University of Nebraska Medical Center

TRANSFORMATION OF MAMMALIAN CELLS BY EXOGENOUS DNA

By

George E. Farley

A THESIS 1969

Presented to the Faculty of

The College of Medicine in the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Medicine

Under the Supervision of Wayne L. Ryan

Omaha, Nebraska

February 3, 1969

TRANSFORMATION OF MAMMALIAN CELLS BY EXOGENOUS DNA

The phenomenon of transformation was observed by Griffith (1) in 1928. He demonstrated that living cells of Pneumococcus Type II (R) plus heat-killed Type III (S) cells produced a fatal infection in mice when injected subcutaneously into mice. Living Pneumococcus Type III (S) cells could later be grown in pure culture from the dead mice. Griffith suggested that the dead bacteria in the inoculum might furnish some specific protein that served as a "pablum" and enabled the R forms to manufacture a capsular material.

The phenomenon of transformation was first observed by Griffith using pneumococcus (1). Later Avery (2) isolated the transforming substance in Griffith's experiment. Further observations by others confirmed the transformation in pneumococci and also showed it to occur in many other bacteria. Since then much work has been done to attempt to show transformation in other microbial groups. In recent years many have tried to extend this work with bacteria to mammalian cells.

Transfer of genetic material by transformation can be defined as "the integration with the genome of a recipient cell of a small piece of exogenous genetic material, extracted from a donor cell and introduced into the receptor as part of a free DNA particle" (3). The operational meaning is a heritable change in a recipient cell produced by highly polymerized DNA extracted from donor cells bearing the new character. "This phenomenon bears no obvious relationship to the term 'transformation' which has been used widely in the tissue culture literature and has been applied to heritable changes in the growth pattern of fresh cell explants and similar changes caused by viral infection" (4).

The nature of the transforming substance alluded to by Griffith was later characterized by Avery (2) in 1944. Avery found that DNA extracted from a smooth, unencapsulated strain of Pneumococcus Type III would transform rough, unencapsulated cells of Pneumococcus Type II into fully capsulated Type III cells. Repeated culture of these cells revealed that this property was passed on from generation to generation. It appeared that Pneumococcal DNA was taken up by the R form Type II Pneumococcal cells and was incorporated into the DNA of these cells. It then provided information for the production of capsular material.

Not only was Avery able to demonstrate the transforming factor, but he also gave impetus to research dealing with DNA. At the time of his experiment it was felt that DNA was a monotonous polymer of a tetranucleotide unit with little specificity. This work by Avery lent credence to the concept of specific differences in DNA. This concept of specific differences has been shown to be valid. The chemical analysis by Chargaff (5) disproved the supposedly uniform composition of DNA. This concept of variation in DNA has been confirmed and affirmed by many experiments and investigators by both biological and chemical means.

Transformation by exogenous DNA has been shown to occur in other bacteria. Strains of H. influenzae (6), streptococci (7), B. subtilis (8), E. coli (9), Neisseria catarrhalis (10), and others have demonstrated this (11). Conditions for transformation vary from species to species and are often very specific (12).

Transformation has not been demonstrated in cells other than bacteria. Failure has resulted from attempts to show transformation in Neurospora and Penicillium.

The successful experiments showing transformation in bacteria spurred attempts to demonstrate it in mammalian cells. Benoit (13) injected DNA extracted from the genital cells and erythrocytes of male Khaki Campbell ducks into the intraperitoneal cavity of 7 day old Pekin ducklings. They had hoped to find changes in the progeny of these treated ducks but were surprised to note somatic changes in the treated ducks. Changes were noted in the head and body type, bill pigmentation and color, and in pigmented spots on the webbing. These changes were noted in nine of twelve treated ducks but in none of the 24 controls. Observations of the progeny have shown these characteristics to be passed on to the third generation. This experiment has been repeated by Benoit (14) and others (15) but no one has been able to reproduce the results noted above.

Attempts to transform the mammals in vivo have been unsuccessful. Tigyi (15) was unable to show somatic changes using DNA from liver and testes of chinchilla and rabbits. Kok (16) failed to show pigment changes in mice using DNA injected into sucklings.

Perry (17) failed to show somatic changes in the rat using DNA injected intraperitoneally, Bearn (18) failed to show pigment changes in the albino rat using

DNA from liver, spleen, and testes injected intraperitoneally as well as into the uterine cavity at the blastocyst stage. Bearn (19) failed to show malignant changes in rats after intraperitoneal injections of DNA from rat sarcoma and hepatoma. Beatty (20) failed to show changes in the rabbit. Yoon (21) attempted to explain the failure to show transformation changes in mice. As can be seen, most attempts at transformation in mammalian cells using DNA injected into the intact animal have met with failure.

Martinovitch (22) injected DNA into the vitelline vein of chick embryos. This caused teratologic changes in the developing chicks with alterations in the feather color. Formation of a modified hemoglobin was also noted in these chicks.

The difficulties and mostly negative results found in the in vivo approaches stimulated attempts to demonstrate transformation using in vitro experiments. In vitro experiments more closely parallel the bacterial experiments and suggested a greater probability of success.

Among the first of these experiments was the work of Kraus (23). DNA isolated from bone marrow of a human homozygous for hemoglobin A was added to marrow cells from

a patient with sickle-cell anemia. The hemoglobin present was homozygous hemoglobin A. From the results of the experiment, it appeared that a modified hemoglobin had been produced. The sickle cell marrow originally produced only alpha^{A}, beta^{S}, and gamma polypeptide chains of hemoglobin but after the addition of DNA from cells capable of producing alpha^{A}, beta^{S}, and gamma chains, these sickle cells were able to produce the alpha^{A}, beta^{S}, and gamma chains in addition to beta^{A} chains. Since the ability to produce beta^{A} chains was absent from the sickle cells, it appeared the exogenous DNA had altered the genetic machinery of the sickle cells giving these cells the information to produce beta chains.

Szybalska (24) worked with tissue culture of a human cell line lacking the enzyme inosine pyrophosphorylase. When treated with DNA from enzyme-plus strains, the experimental cells lacking the ability to produce inosine pyrophosphorylase were able to produce enzyme-containing cells to a statistically significant extent. He was able to demonstrate this because he was able to develop highly selective conditions to detect the transformed cells. Bradley (25) cultivated <u>in vitro</u> cells sensitive to alpha-azaguanine with DNA from cells highly

resistant to alpha-azaguanine. The DNA treated cells yielded appreciably larger numbers of resistant cells than did controls.

Investigation subsequently centered on the uptake of DNA by mammalian cells and on the fate of the ingested DNA. Gartler (26) showed that human tissue culture cells were able to take up labeled DNA. However, he was unable to determine if the DNA was taken up intact or if it were broken down to precursors.

Chorazy (27) demonstrated uptake of radioactive DNA by Ehrlich ascites cells. His work suggested that large fragments of DNA were taken up. King (28) demonstrated uptake of labeled DNA by Strain L fibroblasts. Gartler (29) later used DNA with heavy atom of thymidine C^{14} added so that CsCl density gradient could be used to separate donor from recipient DNA. This indicated that DNA was taken up by Earle's C cells. A small amount of intact DNA was taken up and a smaller amount was degraded. Borenfreund (30) also showed uptake of DNA using HeLa cells. He could not determine if the DNA was degraded or taken up intact. Sirotnak (31) showed uptake of DNA by mouse lymphoma cells. Wilczok (32) demonstrated uptake of DNA by the Novikoff Hepatoma cells. Others have also demon-

strated the ability of cells to take up exogenous DNA(33-39).

Bensch (40) used indigestible gold particles incorporated into the DNA containing coacervates to follow the course of ingested DNA. By use of electron microscopy he was able to follow the breakdown of the DNA. Lysosomes were labeled with saccharated colloidal iron prior to phagocytosis of DNA. Phagocytosis of the labeled coacervates ensued immediately after addition of the particles to the tissue culture. The phagocytosis vacuole containing the DNA merged with digestive vesicles originating in the Golgi apparatus. The result in this experiment using Strain L (Earle) cells and DNA particles prepared by the process of coacervation of the nucleic acid with gelatin was substantial hydrolysis of the ingested DNA.

The above work by Bensch presents interesting information dealing with breakdown of coacervates of DNA. It is difficult to generalize from this specific experiment to other related experiments. Differing uptakes of undenatured DNA and denatured DNA have been shown (41). This may well hold for hydrolysis of denatured DNA and undenatured DNA. The method of preparation and handling of the DNA may be of great importance concerning the

uptake and fate of DNA. The work by Bensch provides a stimulus to attempt to follow the fate of ingested DNA.

Two interesting examples of ability of DNA to resist hydrolysis after intracellular uptake are known. Viral DNA and RNA are able to enter a cell and resist the digestive action of the lysosomes. It appears to be able to escape intact from the digestive vesicles. It may be this ability to escape hydrolysis that determines the infectivity of these viruses. The other example is the penetration of an ovum by the sperm. Here DNA from the sperm migrates through cytoplasm of the ovum and combines with the DNA already present. The DNA is not hydrolyzed but remains intact and competent.

In examining the <u>in vivo</u> attempts at transformation it becomes clear that most have resulted in failure. Various suggestions have been made to explain this.

(1) The biological activity of DNA may have been destroyed in the isolation.

(2) The DNA injected into the animal may have failed to reach the interior of the cells where it could act.

a. The DNA would have to cross several membranes to reach the interior of the cell.

b. The injected DNA may have been destroyed by DNAase in the blood stream.

(3) DNA may not be taken up by mammalian cells.

(4) The DNA taken up by these cells may be destroyed by intracellular enzymes.

(5) In order to see the results of transformation many cells would have to be transformed. Hence if only a few cells picked up the DNA, the transformation might not be apparent.

The bacterial and somewhat analogous in vitro experiments do not suffer from many of these problems. Examination of the areas of difficulty presented suggested that modification of experimental design might enhance the chances of demonstrating transformation in mammalian cells.

After examination of the historical background and previous experiments, the following experiment was designed and attempted. The experiment was not pursued to a successful conclusion and no results were obtained.

A large number of fertilized viable two-cell ova can be recovered from the fallopian tube (42) of a mouse after injection of HCG and PMS (43,44) and subsequent mating. These ova can be isolated and grown in tissue culture (42,45,46,47). The zona pellucida can be removed

by Pronase (48) to facilitate uptake of macromolecules. The ova can be cultured to the blastocyst stages. They can then be transferred to a pseudopregnant female and will develop and grow until parturation (42).

The culture medium can be modified by adding various components. DNA extracted from the thymus or testes of a second strain of mice could be added to the culture medium (49,50). The two-cell ova will grow and divide until it reaches the blastocyst stage of 50^{+} cells The cells would require nucleic acid for replication of the DNA.

It is hoped that the dividing cells would pick up the exogenous DNA and incorporate this into the DNA of these dividing cells. It would then be passed on from cell to cell so that a large number of cells in the animal would be modified at the time of birth.

This model has several features which would serve to enhance the uptake of exogenous DNA. The DNA extraction procedure has been shown to retain the transforming competence of Pneumococcal DNA. Only the single membrane of the ovum needs to be crossed. The cells are rapidly dividing and would be expected to show uptake of DNA with increased chance for incorporation.

Detection of transformation *in vivo* has been attempted by morphological changes and by chemical changes in the treated animals and progeny. *In vitro* work has allowed usage of hemoglobin analysis and sensitivity to chemicals. In the proposed experiment detection of transformation would be sought by morphological changes but also by immunological changes. Inbred strains of mice do not reject skin grafts among members of the same inbred strain. Thus offspring of a mating of these inbred mice would not reject skin grafts from other members of the group. If DNA were incorporated by the dividing cells in the tissue culture alterations in protein, production would result. The altered protein is associated with alterations in the histocompatability of skin then rejection of skin grafts should occur. This would allow another method for detection of a successful transformation.

DISCUSSION

The understanding of nucleic acids is of fundamental importance. The understanding of its composition and characteristics is needed in understanding how it acts. It is well established that DNA is the genetic material

which passes from generation to generation. It directs the production of protein in the cell. This protein then functions enzymatically and structurally to allow the cell to function. The fine control of this process is occupying much research time now. Here may be the answer to how one cell, the fertilized ova, can divide and diversify and specialize resulting in a bacteria, mouse, or man. One cell contains the information needed to direct the production of brain cells, heart cells, liver cells, etc.

The understanding of how DNA is passed from generation to generation is also of great importance. In bacteria, DNA is passed from generation to generation primarily by binary division. This allows for passing on information to maintain the same characteristics from mother cell to daughter cells but does not allow for variation of characteristics. It is the phenomenon of transformation, transduction, mating, and episomal transfer which allow for variation. In the mammalian system, variation is provided by mating. Transduction has been shown to occur. This allows genetic engineers to introduce exogenous DNA with information different

from the host cell into the host cell. Thus the protein production can be altered. Transformation would also allow for introduction of new instructional material into the host cell.

The significance of transformation in bacterial systems deals with genetic mapping, adding new characteristics to an organism, and elucidation of the fundamental role of DNA. In addition to the above, the extension of transformation within mammalian cells would be of great scientific interest and could be of importance in genetic engineering and study of the cancer process. It has been suggested that DNA from a dying cell could be released and picked up by another cell. Thus more genetic material would be available to this cell. This could then lead to changes in the cell's growth and reproduction leading to a cancerous growth. In the area of genetic engineering, it may prove practical to add DNA with a known base sequence to correct genetic defects.

BIBLIOGRAPHY

1. Griffith, F., "The Significance of Pneumococcal Types" J. Hyg. Cambridge Eng. 27, 113, (1928).

2. Avery, O. T., MacLeod, C. M., McCarty, M., "Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types" J. Exp. Med. 79, 137, (1944).

3. Szybalska, E. H., and Szybalski, W., "DNA Medicated Heritable Transformation of a Biochemical Trait" Proc. Nat. Acad. Sci. 48, 2033, (1962).

4. Schaeffer, P., "Transformation " in The Bacteria, Gunslaus, I. C., and Staner, R. Y., Academic Press, New York, pp. 87-153, (1964).

5. Chargaff, E. in The Nucleic Acids, Chargaff, E., and Davidson, J. N., eds. Academic Press, New York, Vol 1. p. 307, (1955).

6. Alexander, H. E., and Leidy, G., "Induction of Streptomycin Resistance in Sensitive H. Influenzae by Extracts Containing DNA from Resistant H. Influenzae" J. Exp. Med. 97, 17, (1953).

7. Bracco, R. M., Kraus, M. R., Roe, A. S., and MacLeod, C. M., "Transformation Reactions Between Pneumococcus and Three Strains of Streptococci" J. Exp. Med. 106, 247, (1957).

8. Vestri, R., "Transformation by Hybrid DNA in Bacillus Subtilis" Nature, 209, 1154, (1966).

9. Anagnostopoulos, C., "Requirements for Transformation in Bacillus Subtilis" J. Bacteriol. 81, 741, (1961).

10. Alexander H. E., Redman, W., "Transformation of Type Specificity of Meningococci" J. Exp. Med. 97, 797, (1953).

11. Ravin, A. W., "Review of Bacterial Transformation and Molecular Biology" Advances in Genetics 10, 61, (1961).

12. Hotchkiss, R. D., "Transforming DNA from Pneumococcus Procedures" Nucleic Acid Res. 541, (1966).

13. Benoit, J. Leroy, P., Vendrely, R., and Vendrely, C. "Experiments on Pekin Ducks Treated with DNA from Khaki Campbell Ducks" Transactions of the New York Academy of Sciences, ser. II Vol 22, 503, (1960).

14. Svoboda, J., Haskova, V., "Failure to Produce Somatic changes Between Strains of Ducks by Means of Specific DNA" Folia Biol. Prague 5, 402, (1959).

15. Tigyi, A., Bendeczky, I. Lissah, K., "Studies on the Modifying Effect of Isolated DNA in Mammals" Acta Biol. Acad. Sci. Hung. 10, 197, (1959).

16. Kok, I. P.,"The Attempt to Obtain Genetic Transformation in Mice by Means of DNA" Nauk. U.S.S.R., 921, (1959).

17. Perry, T. L., Walker, D., "Failure of DNA to Affect Somatic Transformation in the Rat" Proc. Soc. Exp. Biol. Med. 99, 717, (1958).

18. Bearn, J. G., Kirby, K. S., "Failure of DNA to Produce Pigment Changes in the Albino Rat" Expt. Cell Res. 17, 547, (1959).

19. Bearn, J. G. "An Attempt to Produce Malignant Changes with DNA from Rat Sarcoma and Hepatoma" Nature 184, 824, (1959).

20. Beatty, R. A. Billete, F. S., "Attempted Genetic Transformation in the Rabbit" Proc. Roy. Sci. Edinburgh 68, 83, (1958).

21. Yoon, Chai H., " Basis for Failure to Induce Transformation in Vivo With Exogenous Homologous DNA" J. of Heredity 54, 163, (1963).

22. Martinovitch, P. N., Kanazier, D. T., Knezevitch, Z. A., and Simitch, M. M., "Teratological Changes in the Offspring of Chick Embryos Treated with Tyrode or with Tyrode Plus DNA" J. Embryol. Exp. Med. 10, 167, (1962).

23. Kraus, L. M., "Formation of Different Hemoglobin in Tissue Culture of Human Bone Marrow Treated with Human DNA" Nature 192, 1055, (1961).

24. Szybalska, E. H., Szybalski, W., "DNA Medicated Heritable Transformation of a Biochemical Trait" Proc. Nat. Acad. Sci. 48, 2026, (1962).

25. Bradley, R. R., Roosa, R. A., Law, L. W., "DNA Transformation Studies with Mammalian Cells in Culture" J. Cell Comp. Physiol. 60, 127, 1962.

26, Gartler, S. M.,"Cellular Uptake of DNA by Human Tissue Culture Cells" Nature 184, 1505, (1959).

27. Chorazy, M. R., Baldwin, H. H., Boutwell, R. K., "The Incorporation of Radioactive DNA into Ehrlich Ascites Cells" Fed. Proc. 19, 307, (1960).

28. King, D. W., and Bensch, K. G.,"Incorporation of Intact Heterologous DNA into Strain L. Fibroblasts" Fed. Proc. 19, 308, (1960).

29. Gartler, S. M., "Demonstration of Cellular Uptake of Polymerized DNA in Mammalian Cell Culture" Biochem. Biophys. Res. Comm. 30 , 127, (1960).

30. Borenfreund, E., and Bendich, A., "Study of the Penetration of Mammalian Cells by DNA" J. Biophys. Cyt.9, 81,(1961).

31. Sirotnak, F. M., and Hutchison, D. J., "Absorption of DNA by Mouse Lymphoma Cells" Biochemica et Biophysica Acta 36, 246, (1959).

32. Wilczok, T., "Autoradiographic studies on the Incorporation of Heterologous DNA into the Novikoff Hepatoma Cells" Neoplasma 9, 369, (1962).

33. Hays, E. F., "Attempts to Induce Leukemia by Incubation of Mouse Thymus Cells with Leukemia Cell DNA," Nature 209,1327, (1960).

34. Bensch, K. G., King, D. W., "Incorporation of Heterologous DNA into Mammalian Cells" Science 133, 381, (1961).

35. Kay, E. R., "Incorporation of DNA by Cells of the Ehrlich Lettri Ascited Carcinoma", Transactions of the New York Acad. of Sci. ser II, Vol 28, 726, (1966).

36. Hill, M., Jakukickova, J., "Intercellular Passage of DNA as Revealed in Bone Marrow Autoradiographs" Exp. Cell Res. 26, 541, (1962).

37. Riche, W. O., "The In Vivo Reutilization of Lymphocytic and Sarcoma DNA by Cells Growing in the Intraperitoneal Cavity" J. Cell Biol. 13, 205, (1962).

38. Glick, J. L, "DNA Induced Pigment Production in a Hamster Cell Line" J. Cell Biol. 33, 209, (1967).

39. Hudnik-Plevnik, T. A., Glisin, V. R., Simic, M.M., "Fate of Highly Polymerized Spleen DNA Labeled With P^{32} Injected Intraperitoneally into Rats" Nature 184, 1817, (1959).

40. Bensch, K., G., Gorden, Miller, L., "Cytochemical Studies on DNA Containing Particles Phagocytized by Mammalian Cells," Transactions of the New York Acad. Sci. Ser. II Vol 28,715, (1966).

41. Fox, M. S., "Fate of Transforming DNA Following Fixation by Transformable Bacteria" Nature 187, 1004, (1960).

42. Mintz, B., "Formation of Genetically Mosaic Mouse Embryos and Early Development of Lethal (t12/t12) 'Normal" Mosaics" J. Exp. Zool. 157, 273, (1964).

43. Fowler, R. E., Edwards, R. G.,"Induction of Superovulation and Pregnancy in Mature Mice by Gonadotrophins" J. Endocrin. 15, 374, (1957).

44. Edwards, R. G., Gates, A. H., "Timing of the Stages of the Maturation Divisions, Ovulation, Fertilization, and the First Cleavage of Eggs of Adult Mice Treated with Gonadotrophins." J. of Endocrin. 18, 292, (1959).

45. Brinster, R. L., "A Method for In Vitro Cultivation of Mouse Ova From Two-Cell to Blastocyst", Exp. Cell Res. 32, 205, (1963).

46. Brinster, R. L., Thomson, J. L., "Development of 8-cell Mosaic Embryos in Vitro". Exp. Cell Res. 42, 308, (1966).

47. Brinster, R. L., "Studies on the Development of Mouse Embryos in Vitro" J. Reprod. Fertil. 10, 227, (1965).

48. Mintz, B., "Experimental Study of the Developing Mammalian Egg" Removal of the Zona Pellucida". Science 138, 594, (1962).

49. Zamenhof, S., "DNA" in Methods in Enzymology, D. F. Calewick and No. O. Kaplan eds. Academic Press, New York, Vol. 3, pp. 696-701, (1957).

50. Burton, K., "A Study of the Conditions and Mechanisms of the Diphenylamine Reaction of the Colorimetric Estimation of DNA", Biochem. 62, 315, (1956).