tagenic effect of streptomycin is nonspecific, being directed toward a class of molecules carrying genetic information rather than toward a particular determinant.

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GENETICS OF HUMAN CELL LINES, IV. DNA-MEDIATED HERITABLE TRANSFORMATION OF A BIOCHEMICAL TRAIT

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The phenomenon of DNA-mediated genetic transformation,[1] first recorded for Pneumococcus,[2] and later extended to several other bacterial species and to infectious viral DNA was only recently demonstrated to occur in mammalian cells.[3, 4] That the phenomenon may be more generalized in mammalian systems is suggested by the related observations: the uptake of nucleic acids by mammalian cells,[5–15] and the infectivity of naked nucleic acids isolated from mammalian viruses.[16–18]

Numerous attempts have been made to demonstrate genetic transformation in mammalian cells,* but the systems employed apparently were not sufficiently selective. In the majority of cases the experiments were performed partially or entirely

with intact animals, relying on morphological traits,[19] immunological characters,[20] or drug resistance[21] as markers. Success was claimed only for the last markers,[21] but the systems did not permit adequate substantiation. In an attempt to transform tissue culture cells to aminopterin resistance, no transformants were detected above the background of spontaneous mutants.[15] As for other systems in higher organisms, marginal mention should be made of the claimed genetic transformation of morphological characters in ducks, which still awaits substantiation.[22]

There exist also in the literature reports of various nonspecific actions of DNA on cells of higher organisms, including effects on resistance to viruses,[23] mutagenesis,[24] teratological changes,[25] cytological effects,[26] tumorigenesis,[27] and partial reversal of UV killing.[28] The synthesis of modified hemoglobins by immature human erythrocytes *in vitro* has been interpreted as a specific response to both DNA[29] and RNA.[30]

Reported interactions between mammalian cells include cell fusion[31] and formation of chromosomal hybrids by joint cultivation of two cell lines with morphological chromosomal markers.[32]

With the discovery of highly selective genetic markers in the human cell line D98S,[3, 4, 33] a means became available for detecting genetic transformants with a high degree of resolution, i.e., as few as one per 10^7 cells. A mutational system, which involves the selection of inosinic acid pyrophosphorylase (IMPPase)-positive cells from an IMPPase-negative population, was described earlier.[3] In preliminary experiments utilizing this selective system, genetic transformation was demonstrated for D98 cells.[3, 4] The present communication documents in detail and extends these observations.

Materials and Methods.—Cell lines and culture media: The D98S human cell line, the wild-type strain employed in these studies, was originally derived by single clone isolation from the Detroit 98 normal sternal bone marrow line.[34] The mutant lines, the isolation and properties of which were described in earlier publications,[3, 4, 33] originated as follows: D98/AG is an 8-azaguanine-resistant derivative of the D98S line; D98/AH and D98/AH-2 are 8-azahypoxanthine-resistant derivatives of the D98/AG line; D98/APt is an aminopterin-resistant derivative of the D98S line. HeLa cells and rat ML-2 cells were kindly provided by Dr. G. C. Mueller; Dr. L. Siminovitch contributed mouse embryo and L60 cells.

The standard culture medium was a modified Eagle's basal medium supplemented with 10 per cent horse serum (E_{90} medium), used in conjunction with a 5 per cent CO_2 atmosphere.[33]

Isolation of donor DNA: For the isolation of DNA, the donor cells were suspended in 10 volumes of standard saline-citrate (SSC) (0.15 *M* NaCl + 0.02 *M* Na_3·citrate), and lysed with 2% sodium lauryl sulfate. After adjusting the NaCl concentration to 1 *M*, exhaustive deproteinization was carried out by repeated shaking with a 4:1 chloroform-butanol mixture and centrifugation. The nucleic acids were precipitated by addition of two volumes of 95% ethanol, spooled on a glass rod, and redissolved in 5 ml of 0.015 *M* NaCl + 0.02 *M* Na_3·citrate, after which the NaCl concentration was readjusted to 0.15 *M*. Some preparations were rendered RNA-free by treatment for 2 hr (37°C) with 50 μg/ml RNase (heated to 100°C for 10 min), followed by deproteinization and two more precipitations with ethanol.

Procedure for transformation (adopted on the basis of experiments described in subsequent sections): For the preparation of the recipient cell suspension, 4- to 5-day old, heavily seeded cultures were grown up in 4-oz prescription bottles. The cell sheet was rinsed twice with balanced salt solution (BSS) (8.0 gm NaCl; 0.4 gm KCl; 0.35 gm $NaHCO_3$; 1.0 gm glucose; per liter of water) and exposed 4–6 min to 0.25% pancreatin in BSS. The cells were detached from the glass by knocking the bottle against a hard surface and suspended in 3 ml (per bottle) of phosphate-buffered saline (PBS) (7.0 gm NaCl; 0.4 gm KCl; 2.75 gm Na_2HPO_4; 0.25 gm $NaH_2PO_4 \cdot H_2O$; 1.0 gm glucose; per liter of water) supplemented with 50 μg/ml spermine·HCl. The cell con-

centration of the combined suspension was then adjusted to 500,000 cells/ml by diluting with PBS.

To 1.5 ml aliquots of the recipient cell suspension the appropriate amounts of DNA solution and/or PBS were added, bringing the total volume to 2.0 ml/tube. After a 15-min period (37°C), the contents of each tube were distributed between five 60 mm plastic petri dishes containing 5 ml of HAT medium (E_{90} medium + 5 μg/ml hypoxanthine, 0.1 μg/ml aminopterin, and 5 μg/ml thymidine). Following 12–14 days of incubation, with medium changes every 2 to 3 days, the plates were rinsed with PBS, and the colonies were fixed, stained, and counted.[33] Cell viability (plating efficiency) during the DNA treatment was determined at intervals by plating 0.02 ml samples of the reaction mixture in E_{90} medium and scoring the colonies after 7 days incubation.

Results and Discussion.—Conditions for transformation: In developing the standard procedure for assaying transformation in D98 cells, guidance was provided by the experience of other investigators in related fields, including bacterial transformation, and infectivity and uptake of nucleic acids in mammalian systems. Consideration was given to the following five aspects:

(1) *The selective system* employs the IMPPase-positive D98S or D98/AG line as DNA donor and the IMPPase-negative D98/AH-2 line as recipient.[3, 4] Highly selective conditions for the scoring of IMPPase-positive transformants are provided by a medium containing 0.1 μg/ml aminopterin, as inhibitor of *de novo* purine synthesis, 5 μg/ml thymidine, to counteract the accompanying block in thymidylate synthesis, and 5 μg/ml hypoxanthine, as sole purine source (HAT medium). Under these conditions the IMPPase-positive donors are able to form colonies with normal plating efficiency and growth rate, whereas the IMPPase-negative recipient is completely inhibited, being unable to utilize hypoxanthine even at 100 times higher concentrations. Another feature of this selective system, contributing to its high resolution, is the nonmeasurable rate of spontaneous mutation (less than 10^{-7}) towards capacity to grow on the selective HAT medium exhibited by the receptor strain, D98/AH-2, which was chosen on this basis.

(2) *The isolation of transforming DNA* was conducted under conditions similar to those routinely applied in this laboratory for the preparation of *Bacillus subtilis* DNA of high specific transforming activity. The procedure is outined in *Materials and Methods.*

(3) *The competence of the recipient cells,* as measured by the proportion transformed (transformant frequency) at saturating DNA concentrations, was reasonably constant under the conditions outlined in *Materials and Methods.* Therefore, no systematic evaluation of the effect of physiological state on competence was carried out, especially since the routinely prepared recipient cells satisfied the requirement for high plating efficiency.

(4) *The exposure of recipient cells to donor DNA* was carried out under conditions designed to be optimum for both cell survival and DNA uptake. The medium satisfied the following requirements: (*a*) freedom from serum, a source of nucleases; (*b*) physiological osmotic environment and effective buffering capacity, as provided by PBS; (*c*) the presence of a polybasic component, known to enhance the cellular uptake and inhibit the enzymatic degradation of nucleic acids.[9, 14, 35] Both glass-attached and suspended cells were employed as recipients, but since treatment of cells in suspension proved more practical and permitted greater accuracy it became the standard procedure.

During periods of exposure not exceeding 30 min, the survival of the recipient

cells suspended in PBS was 90 to 100%. Therefore, protective additives, such as glycerol, were found unnecessary. When DNA was present in concentrations exceeding 10 μg/ml, viable cell losses of 10 to 15% at 15 min and 20 to 30% at 30 min were encountered. Toxic effects of DNA have been reported by others.[24, 36] The optimum pH range was determined to be 7.0 to 7.5. Below pH 6.5 the transformant yield was sharply reduced.

The addition of 50 μg/ml spermine·HCl was found not to have any adverse effect on cell viability and appeared to be essential to the transformation process (see Table 1). Protamine was tried as an alternative, but at the concentrations em-

TABLE 1

DNA-MEDIATED GENETIC TRANSFORMATION IN D98 CELLS: TRANSFER OF CAPACITY TO UTILIZE HYPOXANTHINE FROM IMPPASE-POSITIVE DONORS TO IMPPASE-NEGATIVE D98/AH-2 RECIPIENT CELLS

DNA donor	IMPPase activity of donor	Treatment of donor DNA	No. of Transformants/ml* Donor DNA concentration (μg/ml) 0	1	10	100
D98S	+	...	0	1	32	128
D98/AG	+	...	0	2	19	62
D98/AH	−	...	0	..	0	0
D98/AH-2	−	...	0	..	0	0
D98/AG	+	RNase†	..	..	..	65
D98/AG	+	DNase‡	..	..	..	0
D98/AG	+	spermine omitted	..	..	0	0

* Assayed under standard conditions, as described in *Materials and Methods*, in presence of 50 μg/ml spermine·HCl except where indicated.
† Treated with heated (10 min, 100°C) RNase preparation (50 μg/ml) for 30 min at 37°C.
‡ Treated with DNase preparation (2 μg/ml) for 30 min at 37°C in presence 10^{-2} *M* Mg^{++}.

ployed it caused precipitation of the DNA. Application of osmotic shock, for enhancing the uptake of the nucleic acid by the recipient cells,[8, 37] was found impractical, since exposure of D98 cells to 1 *M* NaCl even for brief periods proved detrimental to cell viability.

Recipient cells were allowed to react with donor DNA for 5–60 min prior to plating. The maximum ultimate yield of transformants was reached between 5 and 10 min; beyond that time no further increase occurred. It has been reported that the absorption of DNA by mammalian cells, as with bacteria, is a very rapid process.[7, 13]

(5) *The phenotypic expression period* in the transformation process was analyzed by plating and preincubating DNA-treated recipient cells for periods of up to 4 days in nonselective medium prior to imposition of the selective conditions. Since the yield of transformants was independent of the length of the preincubation, and since large populations of D98/AH-2 cells were observed to survive attached in HAT medium for 3–4 days, it was concluded that integration of the genetic determinant and its phenotypic expression was accomplished well within this period.

Specific role of donor DNA in transformation: The specific role of donor DNA in the transformation process was established by the following observations: (1) Transformants are produced only in response to a specific DNA, isolated from IMPPase-positive strains, while DNA isolated from IMPPase-negative cells is inactive (Tables 1 and 2). (2) DNase completely destroys the transforming activity, while RNase action is without effect (Table 1). (3) The yield of transformants is a

TABLE 2

CHARACTERISTICS OF TWO TRANSFORMANT LINES AS COMPARED WITH RECEPTOR STRAIN, DNA DONORS, AND SPONTANEOUS "REVERTANT"

	Recipient D98/AH-2	DNA donors D98S	DNA donors D98/AG	Spontaneous "revertant" D98/AH-R	Transformants D98/AH-TS (Donor: D98S)	Transformants D98/AH-TAG (Donor: D98/AG)
Sensitivity to purine analogs:*						
AG	400	0.12	12	100	0.2	0.15
AH	1800	0.12	0.28	80	0.13	0.09
TG	12	0.006	0.12	8	0.01	0.008
Capacity to utilize hypoxanthine†	>500	0.08	0.5	0.1	0.15	0.07
IMPPase activity‡	−	+++	+++	+	+++	...
Transforming activity of isolated DNA:§						
4 μg/ml	0	8	9	...	12	...
40 μg/ml	0	102	48	...	92	...

* Concentration (μg/ml) permitting 50% survival (colony formation); AG = 8-azaguanine, AH = 8-azahypoxanthine, TG = 6-thioguanine.

† Concentration of hypoxanthine (μg/ml) permitting 50% survival in presence of 0.02 μg/ml aminopterin and 5 μg/ml thymidine.

‡ Assayed with cell-free enzyme preparations in presence of hypoxanthine-C^{14} and PRPP.

§ Transformants/ml, assayed under standard conditions, as described in *Materials and Methods.*

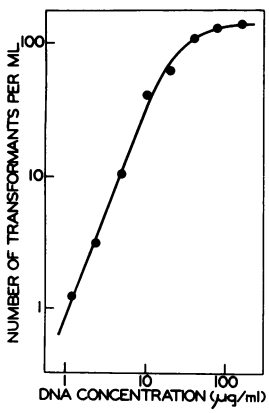

FIG. 1.—Number of IMPPase-positive transformants per ml of "reaction mixture," assayed in HAT medium, following treatment of IMPPase-negative D98/AH-2 recipient cells with increasing concentrations of DNA isolated from IMPPase-positive D98S donor cells, under conditions described in *Materials and Methods.*

function of the DNA concentration, as shown in Table 1 and Figure 1. The response is roughly linear up to about 20 μg DNA/ml, with the curve leveling off at a yield of approximately 150 transformants per ml (4×10^{-4} transformants per recipient cell). (4) DNA and transforming activity sediment in similar fashion in the CsCl equilibrium density gradient, as described in the following section.

Fractionation of transforming principle in the CsCl density gradient: In addition to providing further evidence that DNA and the transforming principle are indeed identical, it was hoped that CsCl gradient centrifugation[38] would permit separation of active fractions from the competing and inactive DNA. Since the specific transforming activity of D98 DNA is low, it was necessary to handle relatively larger amounts than in the analogous bacterial experiments.[39] On the other hand, overloading the centrifuge tube with DNA prevents sharp fractionation. The procedure outlined below is necessarily a compromise between the two requirements.

Approximately 50 μg of DNA in 3 ml of CsCl solution (density 1.69 gm/cm³), overlayered with 2 ml of paraffin oil, was spun for 72 hr in the S39L swinging bucket rotor of the Spinco Model L preparative centrifuge at 35,000 rpm. Five-drop

fractions (approximately 50 μl) were collected as described by Szybalski.[40] OD_{260} was determined for each undiluted fraction, using 20 μl microcuvettes. The fractions were then dialyzed against SSC, since CsCl was found to be toxic in concentrations above 0.05 *M*. The transforming activities (number of transformations per fraction), assayed as described in *Materials and Methods*, are plotted in Figure 2,

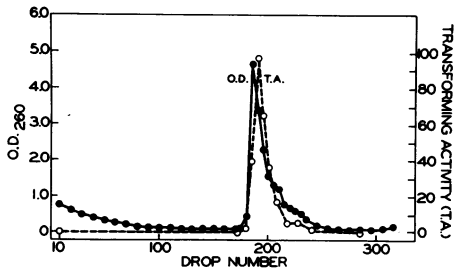

FIG. 2.—Distribution of D98S DNA (O.D.), measured as UV absorbance at 260 mμ and of its transforming activity (T.A.), expressed as the number of IMPPase-positive transformant colonies per fraction, among 50 μl fractions collected after CsCl equilibrium density gradient centrifugation (35,000 rpm; 20°C; 72 hr).

along with the OD_{260} readings. It is apparent that transforming activity sediments in the same buoyant density range as DNA. However, it bands more sharply than the total DNA, and the peak is shifted by one fraction from the peak OD_{260}, indicating partial purification of the active transforming material.

Properties of the transformants: Of several transformant clones isolated and characterized all closely resembled the D98S line, as regards their ability to utilize hypoxanthine and their sensitivity to various purine analogs (see Table 2). Enzymatic assay (kindly performed by Dr. R. W. Brockman and P. Stutts of the Southern Research Institute, Birmingham, Ala.) on one of the transformant lines, D98/AH-TS, revealed normal IMPPase activity, comparable to that of the donor lines, D98/AG and D98S. By the same test, employing C^{14}-labeled hypoxanthine or guanine as substrate in the presence of PRPP, no IMPPase activity could be detected in the receptor strain.[41] The aminopterin sensitivity of the transformants did not differ from that of the receptor and donor lines. During repeated subculture, the transformant lines bred true, with respect to the above properties, indicating the heritable nature of the change. DNA was isolated from the transformant line, D98/AH-TS, and its transforming activity proved to be comparable to that of the other IMPPase-positive donors, D98S and D98/AG (see Table 2).

The transformants could readily be distinguished from the spontaneously occurring "revertant," D98/AH-R, which utilizes hypoxanthine efficiently but shows intermediate degrees of resistance to hypoxanthine and guanine analogs and only a very low IMPPase activity (see Table 2). This "revertant," described earlier,[3, 4] can be isolated from D98/AH populations, although not from the D98/AH-2 line, employed as recipient in the transformation studies.

Transforming activity of heterologous DNA's and interference by nontransforming DNA's: The observation that IMPPase-positive transformants are produced only in response to DNA isolated from IMPPase-positive D98 donors lead to two further questions. (1) Would heterologous DNA's isolated from other human cell lines and from unrelated mammalian sources known to possess an active IMPPase be effective as the transforming agent; and (2) would genetically inactive DNA's interfere with the transformation process, in analogy with the interference phenomenon observed in bacterial transformation?

(1) Highly polymerized DNA was isolated from the HeLa cell line of human malignant origin, from the ML-2 rat cell line, and from mouse embryo and L60 cells. As can be seen in Table 3, HeLa DNA appeared to have a very low transforming activity, while the mouse and rat DNA's were inactive.

(2) The effect of nontransforming homologous (D98/AH) DNA and of heterologous human (HeLa) and mouse (L60) DNA on transformations initiated by D98S donor DNA at near saturation level was determined. As indicated in Table 3,

TABLE 3

TRANSFORMING ACTIVITY OF HETEROLOGOUS DNA'S AND INTERFERENCE BY NONTRANSFORMING DNA'S

	No. of Transformants/ml*							
		D98/AH DNA		HeLa DNA		Mouse L60 DNA	Mouse Embryo DNA	Rat ML-2 DNA
D98S DNA concentration	—	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml	50 μg/ml	50 μg/ml	50 μg/ml
0 μg/ml	0	0	0	0	2	0	0	0
10 μg/ml	52	26	15	35	27	30	..	..

* Assayed under standard conditions, as described in *Materials and Methods.*

transformation was markedly inhibited by D98/AH DNA at a concentration equal to that of donor DNA, and the effect was magnified when the ratio of interfering DNA to donor DNA concentration was increased. Similar effects were observed for HeLa DNA and L60 DNA.

Attempted transformation in other systems: The experiments described above were based on selection of IMPPase-positive transformants from an IMPPase-negative recipient population in HAT medium (D98/AH → D98S). Other selective systems available for D98 cells include: (1) from sensitivity to 100-fold resistance to 8-azaguanine (D98S → D98/AG); and (2) from sensitivity to over 5,000-fold resistance to 8-azahypoxanthine (D98S, D98/AG, or D98/AH-TS → D98/AH).[3, 4, 33] The first system was handicapped by a high spontaneous background of resistant cells (10^{-3} to 10^{-4}) and was therefore abandoned. In the second system no transformations were detected, even though periods as long as 5 days were allowed to elapse between plating of the DNA treated cells and addition of the selective agent.

Attempted genetic recombination between intact cells: A preliminary test for genetic interaction between intact cells was carried out, employing two singly marked mutants, D98/AH, resistant to more than 1,000 μg/ml 8-azahypoxanthine, and D98/APt, resistant to 0.1 μg/ml aminopterin. A 4-oz prescription bottle was seeded with a mixed inoculum of approximately 10^6 D98/AH and D98/APt cells. This culture was carried in E_{90} medium for a total of 14 days with one transfer after 7 days. On the 7th and 14th days, 10^6 cells of this mixed culture were plated in a doubly selective medium containing 10 μg/ml 8-azahypoxanthine and 0.02 μg/ml aminopterin, concentrations completely inhibitory to lines D98/APt and D98/AH respectively. No recombinants were produced, since no colonies appeared in this medium.

Summary.—The treatment of the IMP-pyrophosphorylase (IMPPase)-deficient human cell line, D98/AH-2, with DNA isolated from IMPPase-positive D98S or D98/AG cells resulted in the appearance of IMPPase-positive genetically transformed cells, which were detectable under highly selective conditions. DNAse

but not RNAse treatment abolished the transforming activity. The yield of transformants was a linear function of the transforming DNA concentration up to the level of about 20 μg DNA/ml, reaching a plateau of approximately 4×10^{-4} transformations per recipient cell at 150 μg DNA/ml. Transforming activity banded in the CsCl equilibrium gradient more sharply than the total DNA mass. DNA isolated from the D98/AH-2 recipient cells or from the cells of other mammals did not transform *per se*, but interfered with the active transformation process. No recombinations between intact cells were detected, using aminopterin and 8-azahypoxanthine resistance as selective markers.

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The following abbreviations are used in this paper: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DNase, deoxyribonuclease; RNase, ribonuclease; IMP, inosinic acid; IMPPase, inosinic acid pyrophosphorylase; PRPP, α-5-phosphoribosyl-pyrophosphate; UV, ultraviolet light; OD_{260}, optical density at 260 mμ (1 cm light path).

* The term genetic transformation is used here with the same operational meaning implied as in bacterial genetics, i.e., a heritable change in the 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; cf. Swim, H. E., *Ann. Rev. Microbiol.*, **13**, 141 (1959), and Shein, H. M., and J. F. Enders, these PROCEEDINGS, **48**, 1164 (1962).

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*A CRITERION FOR THE EXISTENCE OF INERTIAL BOUNDARY LAYERS IN OCEANIC CIRCULATION**

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1. *Introduction.*—Inertial boundary layers play an essential role in recent theories of oceanic circulation.[1-3] These phenomena are examined anew in this paper from a rather general viewpoint. The basic objective is the derivation of a relatively simple criterion for the occurrence of such boundary layers in an ocean, of arbitrary shape, whose interior or primary circulation results (wholly or in part) from an existing wind-stress system.