

DNA-INDUCED PIGMENT PRODUCTION IN A HAMSTER CELL LINE

J. LESLIE GLICK and ANTHONY P. SALIM. From Roswell Park Memorial Institute, Buffalo, New York

A number of workers have demonstrated uptake of DNA by mammalian cells (1-6). At least some of the donor DNA appears to be incorporated intact into the nuclei of the host cells (3-6). However, very few investigators have reported genetic transformation of mammalian cells by mammalian DNA (7-9). Two cases which did indicate genetic transformation depended upon measuring the growth of drug-sensitive cells in the presence of drug, after they had been treated with DNA from drug-resistant cells (7, 8). A third study of genetic transformation involved an electrophoretic analysis of hemoglobin peptide chains in sickle cells which had been treated with DNA from normal bone marrow cells (9). In this study, we have attempted to induce the appearance of pigment in amelanotic melanoma cells with DNA obtained from melanotic melanoma cells.

MATERIALS AND METHODS

Hamster cell lines of MM2 melanotic melanoma (RPMI #3460) (10), AM5 amelanotic melanoma (RPMI #7113) (11), and SB1 small bowel carcinoma (RPMI #2402) were grown in McCoy medium plus 20% fetal calf serum, McCoy medium plus 10% fetal calf serum, and medium RPMI #1640 plus 5% fetal calf serum, respectively. The doubling times of these cell cultures were about 45 hr for MM2, 30 hr for AM5, and 15 hr for SB1. Both of the melanoma lines grew as confluent cell sheets in Roux culture bottles. The SB1 line was maintained in suspension culture. All three cell lines grew as solid tumors in Syrian hamsters, but only the MM2 tumors contained brown, melanin pigment granules. This pigment was also found in MM2 cells which had been cultured *in vitro* for periods as long as 1-3 yr (G. E. Moore, personal communication). The percentage of MM2 cells which developed pigment in any given culture varied with the state of growth of the culture. Only 30% of the MM2 cells might contain pigment in a fresh subculture, but over 90% of the cells could develop pigment within a week. In contrast, a maximum of only 0.1-0.3% of AM5 cells appeared to contain pigment at any given time. SB1 cells never developed pigment.

DNA was isolated from MM2 cells and SB1 cells by the method of Szybalska and Szybalski (7). The DNA preparations were treated with ribonuclease

and were repurified before use (7). Whereas SB1 DNA was obtained from cells grown *in vitro*, MM2 DNA was obtained from a solid tumor which developed in a hamster injected with cells grown *in vitro*. This latter procedure for harvesting large quantities of MM2 cells was necessary, because these cells grow slowly as monolayer and hardly proliferate in suspension culture.

The technique used for treating cells with DNA was that of Szybalska and Szybalski (7). AM5 or SB1 cells were washed free of growth medium and were incubated with or without DNA in a sterile, phosphate-buffered (pH 7.5) saline solution (7) containing 5.5 mM glucose and 0.2 mM spermine. All incubations lasted 20 min at 37°C. The cells were washed and then were resuspended in the appropriate growth media listed above. Each group of cells was diluted to 1.7×10^5 cells/ml, and aliquots of 3.0 ml each were added in triplicate to Earle's T-15 culture flasks. The flasks were then kept at 37°C.

Cells were observed from 1 to 6 days after DNA treatment, by the method of inverting the flasks under a microscope. Five areas, about 0.5-1.0 cm each, were arbitrarily circled on each flask. The cells which adhered to the glass surface were counted in each circled area; very few cells were found detached from the surface. Since each group of cells was divided into three flasks, a total of 15 areas per group was checked each day. The flasks were coded to prevent any bias on the part of the observer in distinguishing pigmented from nonpigmented cells. The percentage of pigmented cells in each group was determined in two ways. The first procedure merely involved totaling the numbers of pigmented and nonpigmented cells in all 15 areas and then obtaining the over-all percentage of pigmented cells. Statistical significance among the different groups was determined by means of chi square analysis. The second method involved calculating the percentage of pigmented cells in each of the 15 areas and then finding the mean percentage of pigmented cells for each group, \pm the standard error of the mean. Statistical significance among the different groups was then determined by means of the *t* test.

In one experiment, the cloning technique of Schindler (12), as modified by Ito (E. Ito, personal communication), was employed in an attempt to isolate the progeny of pigmented cells in petri dishes. By means of this procedure, AM5 cells which had been preincubated with or without DNA were grown

TABLE I
Effect of DNA Preparations Obtained from Different Sources on Pigmentation of AM5 Cells

Treatment of cells	Percentage of pigmented cells	
	Chi square analysis	t test analysis
Saline-citrate	0.15 (18/11689)	0.16 ± 0.05
SB1 DNA	0.19 (20/10653)	0.15 ± 0.06
MM2 DNA	0.44 (44/10076)*	0.45 ± 0.12‡

The concentration of cells during the various treatments was 1.8×10^6 /ml. The DNA concentration was $60 \mu\text{g}/10^6$ cells. The total volume of the incubation medium was 4 ml. The above results were obtained from cell counts 3 days after DNA treatment. Values in parentheses are numbers of pigmented cells per total number of cells. See text for further explanation.

*P < 0.001 compared to saline-citrate treatment; P < 0.01 compared to SB1 DNA treatment.

‡ P < 0.05 compared to each of the other treatments.

in fibrinogen-thrombin gels containing McCoy medium plus 10% fetal calf serum. The cells were diluted to about 100 per petri dish, and the dishes were placed in a CO₂ incubator. Ten dishes contained saline-citrate-treated cells, four dishes contained SB1 DNA-treated cells, and ten dishes contained MM2 DNA-treated cells.

RESULTS AND DISCUSSION

Tables I and II demonstrate that MM2 DNA enhanced the percentage of pigmented AM5 cells threefold, whereas neither SB1 DNA nor deoxyribonuclease-treated MM2 DNA had any effect. The net increase in the percentage of pigmented cells induced by MM2 DNA was about 0.3% for $60 \mu\text{g}$ of DNA per 10^6 cells (Table I) and about 0.6% for $90 \mu\text{g}$ of DNA per 10^6 cells (Table II). Since small percentages of AM5 cells are normally pigmented, the results were subjected to two types of statistical analysis. The chi square method established a significant difference in the over-all percentage of pigmented cells between each of the MM2 DNA-treated groups and each of the other groups. The t test demonstrated that the individual percentages of pigmented cells were relatively constant among the 15 areas scanned per group, so that the mean percentage of pigmented cells was significantly higher in each of the MM2 DNA-treated groups than in the other groups. In order to demonstrate that the induction of pigment in

AM5 cells by MM2 DNA was a stable, heritable change, we studied the growth rates of MM2 DNA-treated cells. Fig. 1 shows that the newly pigmented cells grew logarithmically like the total population of cells, without loss of pigment granules. In this experiment, the mean generation time was 36 hr for both pigmented and nonpigmented cells.

When AM5 cells were cloned, only about 18% of the cells grew in each petri dish. Cell division continued up to 2 wk after initiation of plating, but cell clusters were hardly noticeable macroscopically. Locating cell clumps microscopically was both tedious and difficult, since one had to focus continuously on different levels of the fibrinogen-thrombin gel. Over 250 cell clumps were obtained in petri dishes containing either saline-citrate-treated cells or SB1 DNA-treated cells, but none of the clusters appeared to contain any pigmented cells. There were about 180 clusters of MM2 DNA-treated cells, however, and at least one of these clumps consisted of 25 definitely pigmented cells, 1 wk after cloning.

Studies with MM2 DNA-treated SB1 cells were entirely negative with respect to induction of

TABLE II
Effect of Deoxyribonuclease (DNase)-Treated MM2 DNA on Pigmentation of AM5 Cells

Treatment of cells	Percentage of pigmented cells	
	Chi square analysis	t test analysis
Saline-citrate	0.27 (21/7862)	0.24 ± 0.06
DNase	0.22 (17/7686)	0.26 ± 0.13
DNase + MM2 DNA	0.29 (20/6849)	0.25 ± 0.09
MM2 DNA	0.82 (55/6697)*	0.85 ± 0.20‡

The concentration of cells during the various treatments was 9.3×10^5 /ml. The DNA concentration was $90 \mu\text{g}/10^6$ cells. The total volume of the incubation medium was 3.5 ml. DNase degradation of MM2 DNA was performed according to a previously established technique (7). The above results were obtained from cell counts 1 day after DNA treatment. Values in parentheses are numbers of pigmented cells per total number of cells. See text for further explanation.

* P < 0.001 compared to each of the other treatments.

‡ P < 0.01 compared to saline-citrate treatment; P < 0.05 compared to DNase treatment; P < 0.02 compared to DNase + MM2 DNA treatment.

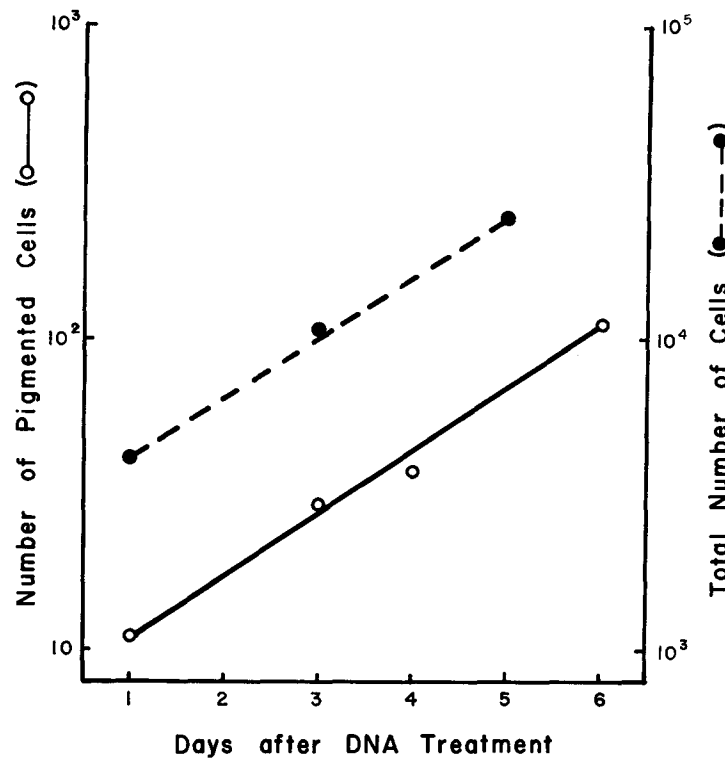


FIGURE 1 Growth rates of MM2 DNA-treated AM5 cells. The total number of cells includes both pigmented and nonpigmented cells. The number of pigmented cells is corrected for the number of pigmented cells (0.2%) found in an equivalent population of saline-citrate-treated cells. Incubation conditions same as in Table I.

melanin pigment. No pigment granules were observed in 5605 saline-citrate-treated cells, 5787 SB1 DNA-treated cells, or in 7455 MM2 DNA-treated cells, 1 day after treatment. No pigment granules were observed even 3 days after treatment, when 15,000–20,000 cells were scanned in each flask. The lack of effect of MM2 DNA on SB1 cells perhaps reflected the greater disparity between MM2 and SB1 genotypes than between MM2 and AM5 genotypes. Presumably, more than one MM2 gene would have to be inserted into the SB1 genome in order to induce pigmentation, so that the likelihood of observing any phenotypic change would be negligible by means of the above experimental procedure.

In conclusion, the above phenotypic change in amelanotic melanoma cells appeared to be induced specifically by melanotic melanoma DNA. The newly formed pigmented cells appeared capable of proliferating as well as nonpigmented cells, so

that the acquired characteristic was transferred readily from one generation to the next, at least for the few cell doublings that were observed. Although the percentage of AM5 cells which were induced was low, it was 10-fold higher than that reported by Szybalska and Szybalski (7) for genetic transformation of aminopterin resistance in D98 cells. Such a difference could be resolved, if AM5 cells are more competent than D98 cells to the penetrating DNA.

SUMMARY

Exposure of amelanotic hamster cells to DNA extracted from hamster melanoma resulted in a 3-fold increase in pigment-producing cells. This effect was not observed if the DNA was pretreated with deoxyribonuclease. Intact DNA from hamster small bowel carcinoma had no effect on pigmentation of the amelanotic cells.

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