# REGULATION OF PIGMENT SYNTHESIS IN MAMMALIAN CELLS, AS STUDIED BY SOMATIC HYBRIDIZATION*

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One of the main problems concerning differentiation in higher organisms is that of its genetic control. Whether positive or negative control mechanisms operate in differentiation, or indeed whether the problem can be stated in these terms borrowed from bacterial genetics, is still unknown. One of the possible approaches to these questions is to determine the effects of combining, through somatic hybridization, the genomes of two cells differing in at least one specific function. An earlier series of experiments along this line,[1] to be discussed below, gave no evidence of interaction between the genomes in hybrid cells.[2] In this communication we report the results of similarly designed experiments, involving melanin synthesis as the specialized function.

The pigment-producing cells used in all the experiments are from a subline of the Syrian hamster melanoma RPMI 3460, described by Moore,[4] into which we have introduced an 8-azaguanine resistance marker. One pigmented clone (3460-3) has been used in most of the experiments. To determine the frequency of amelanotic cells in 3460-3, we have subcloned this population on a large scale. Among the *ca.* 15,000 colonies formed (with a cloning efficiency of nearly 90%), none were unpigmented.

The melanoma cells were hybridized with each of three unpigmented mouse permanent cell lines, each resistant to 5-bromodeoxyuridine. These lines include B 82 and LM(TK$^-$) clone 1D, derived from two different sublines of L cells, and two clones of N 2, derived from NCTC 2555.[5] The karyotypes of the mouse and hamster lines are very different, making the karyological identification of the hybrids very easy. The hamster cells have a modal number of 51 chromosomes, the majority (43) of which are biarmed. All the mouse cells used have modal chromosome numbers of approximately 50, but most of their chromosomes (30–46, depending on the particular cell line) are telocentric.

The method of isolation of the hybrids is based upon the system developed by Littlefield[6] for selecting hybrids between two biochemically marked L cells. Littlefield has shown that neither an 8-azaguanine resistant cell which lacks inosinic acid pyrophosphorylase, nor a 5-bromodeoxyuridine resistant cell which lacks thymidine kinase can grow in a medium containing $1 \times 10^{-4}$ *M* hypoxanthine, $4 \times 10^{-7}$ *M* aminopterin, and $1.6 \times 10^{-5}$ *M* thymidine. The hybrids inherit the synthetic abilities of both parents, and hence can grow in such a selective medium. The same medium has already been used in this laboratory to obtain viable somatic rat × mouse[7] and hamster × mouse[8] hybrids. None of the parental cell lines used in the present experiments grow in the selective medium.

For the mating experiments, $10^6$ cells of one of the mouse lines were mixed with 400–20,000 melanoma cells in 6-cm Falcon plastic tissue-culture dishes containing Dulbecco's modification of Eagle's medium supplemented with 10 per cent calf serum. One day later, the medium was replaced by the above-mentioned selec-

tive medium. After about 2 weeks at 36°C with periodic feeding of cultures, large colonies of cells, morphologically different from the parental cells, were seen. Karyological analysis proved that these cells were hybrids, containing *ca.* 100 chromosomes, with the proportion of metacentric and telocentric chromosomes expected for each particular combination of melanoma and mouse cells. In a manner analogous to the Luria-Delbrück method of determining mutation rates in bacteria,[9] the following "mating rates" were obtained from the per cent of "mating dishes" inoculated with a given number of melanoma cells which did not have any hybrid colonies: one out of every 1000 melanoma cells mates with N2, one out of every 4000 with LM(TK$^-$) clone 1D, and one out of every 9000 with B82. Several colonies of hybrid cells were isolated and subcultured, and at present have undergone up to 100 cell generations without any decline in growth rate.

As indicated earlier, our purpose in producing these hybrids was to establish whether they would express the specialized cell function exhibited by one of the parents. Thus far, under conditions under which melanoma cells become heavily pigmented, the hybrid cells have remained unpigmented.

In an attempt to locate the block to pigment formation, we have determined the dopa oxidase activity of the parental and hybrid cells growing in the maintenance medium. The cells were collected by trypsinization and homogenized in 0.1 *M* phosphate buffer, pH 6.8, containing 0.2 per cent sodium deoxycholate. For the assay, L dopa to a final concentration of 2 mg/ml was added to equal amounts of protein of each of the homogenates. The optical density was determined over a period of 3 hr at 37°C with a Klett-Summerson photoelectric colorimeter, using a blue (no. 42) filter. The results of one of the experiments in which the activities of the parental cells and of one clone of each of the hybrids were determined are given in Table 1. It can be seen that the melanoma cells have high dopa oxidase activity, whereas all the other lines tested have no such activity. (In fact, all the latter cell lines give values less than that obtained for dopa autoxidation, accounting for the negative numbers in the table. This is due to the presence in the cells of a weak inhibitor, which has negligible effect when added to a melanoma extract.)

The absence of pigment and dopa oxidase in the hybrid cells suggested that the genome of the unpigmented parent represses the expression of the melanoma genes involved in pigment synthesis. However, before this interpretation could be accepted, several alternative explanations had to be considered. First, there was the possibility that *all* hamster genes are repressed in the hybrids. This interpretation was disproved by two facts: (*a*) the production by the hybrid cells

TABLE 1

DOPA OXIDASE ACTIVITY OF PARENTAL AND HYBRID LINES

| Cell line | Activity* |
|---|---|
| 3460-3 | 476 |
| B 82 | −18 |
| LM(TK$^-$) clone 1D | −20 |
| N 2 | −21 |
| 3460-3/B 82 | −21 |
| 3460-3/LM(TK$^-$) clone 1D | −18 |
| 3460-3/N 2 | −22 |

* Activity is expressed as the change in optical density produced by 500 μg of protein during the first 3 hr of the assay. All values are corrected for dopa autoxidation (32 in this experiment).

of both hamster and mouse subunits of lactate dehydrogenase, as demonstrated by starch gel electrophoresis; (*b*) the ability of the hybrid cells to grow in the selective medium inhibiting the growth of the parental cells, which indicates that the hamster and mouse genomes complement each other in the hybrids. (This complementation necessarily involves the production of thymidine kinase by the hamster genes, since the mouse cells (B 82,[10] LM(TK$^-$) clone 1D,[11] and, presumably, N 2) lack this enzyme.) These results are in agreement with the demonstration by Weiss and Ephrussi[12] of the continued activity in somatic rat × mouse hybrids of the genes of both parents specifying lactate dehydrogenase and $\beta$-glucuronidase. Second, the possibility had to be considered that the hybrids resulted from fusion of mouse cells with amelanotic hamster cells. This was made very unlikely by the observations mentioned above that one out of every 1000–9000 melanoma cells mates with a mouse cell, while less than one out of 15,000 melanoma cells is amelanotic. Lastly, the possibility that the hybrids had lost their pigment-forming genes appeared improbable, since the decrease in the chromosome number of the hybrids did not exceed 10 per cent. With such a small (and presumably random) loss of chromosomes, the probability of all hybrids losing the same genes is very low. On the basis of these considerations, we are inclined to conclude that a step in the process of pigment formation in the cells studied is under negative control.

This conclusion, if correct, raises several questions: (*a*) Is the repression of the pigment-forming process in the hybrids dependent upon the continued presence of certain genes of the unpigmented mouse cells? (*b*) At what point in this process is the negative control exerted? (*c*) Are other processes associated with the differentiated state of pigment-producing cells affected by hybridization in a similar manner? (*d*) Can our conclusion be extrapolated to the regulation of pigment formation in normal melanocytes? (*e*) Can it be extended to other types of differentiation?

While answers to some of these questions are expected in the near future, the following remarks are appropriate at this time. It should be recalled that two differentiated functions characteristic of another cell type have already been examined in experiments designed similarly to the above.[1] Hybrids between cells of mouse fibroblast lines differing in the rates of collagen and hyaluronic acid synthesis were isolated. The rates of production of these substances by the hybrid cells were found to be intermediate between those of the parents. The difference between these results and those reported in the present paper may indicate the operation in differentiation of a variety of control mechanisms. It may, however, be due as well to differences in experimental design, such as the use of interspecific hybrids in one case and of intraspecific hybrids in the other. Finally, in assessing the relevance of the results of the two series of experiments to differentiation of normal cells, it must be kept in mind that the parents of both sets of hybrids are cells of heteroploid, "established" lines. Therefore, it is possible that neither of the results described above reflects the regulation of differentiation as it occurs in normal cells.

*Summary*.—Hybrids between cells of a Syrian hamster melanoma line and each of three unpigmented mouse lines have been isolated and maintained in active proliferation *in vitro* for up to 100 cell generations. These hybrids have thus far remained unpigmented under conditions under which the melanoma cells become

heavily pigmented, and they exhibit no dopa oxidase activity. It is concluded that a step in the process of pigment formation in the cells studied is under negative control.

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[2] Using UV-inactivated Sendai virus, Harris *et al.*[3] have produced heterokaryons between cells whose nuclei differ in DNA and/or RNA synthesis. Unfortunately, the interactions observed cannot be interpreted in terms of genetic regulation, since they were recorded before dilution of factors present in the cells at the time of fusion could have taken place.

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