Gene Dosage Dependence of Pigment Synthesis in Melanoma x Fibroblast Hybrids

(hamster cells/mouse fibroblast/DOPA-oxidase/irradiation)

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ABSTRACT Hybrids between Syrian hamster melanoma cells and mouse fibroblasts, containing one genome (1s) of each parent, produce neither melanin nor DOPA-oxidase ("extinction"). Attempts to induce loss of the fibroblast chromosomes by irradiation of the fibroblasts before fusion with melanoma cells resulted in the formation of colonies comprising pigmented hybrid cells, which contained 2s melanoma and 1s fibroblast chromosome-complements suggesting that extinction or re-expression of melanogenesis is a function of genetic balance. This interpretation was confirmed by crosses between 2s melanoma cells with unirradiated 1s fibroblasts, which produced both pigmented and unpigmented hybrids. No correlation has thus far been established between karyotype and phenotype of the hybrid cells, but analysis of the karyological data suggests that the fibroblast chromosomes responsible for extinction cannot be numerous.

Among the various findings bearing on the problems of determination and differentiation in mammalian cells, made by means of somatic hybridization, three appear to us of particular importance because they may represent leads for the study of the genetic and molecular mechanisms of these phenomena. The first one of these findings is the extinction of tissue specific ("luxury") functions in hybrids between cells, one of which expresses a given luxury function, the other not. This phenomenon was first observed on hybrids between pigmented producing cells of a Syrian hamster melanoma (3460-3) and mouse fibroblasts of line LM (TK−), Cl. 1D (1). The hybrids resulting from this cross continue to synthesize "household" enzymes (common to all cells) of both species, but they do not produce either melanin or DOPA-oxidase ("extinction"). It was suggested that this may be due to the production by the genome of the fibroblasts of a fusible regulator substance whose final effect is negative.

The extinction of several other luxury functions has now been observed in (intra- and interspecific) hybrids between cells of different histogenetic origins. It must be pointed out, however, that a few exceptions (some of them questionable) to the rule of extinction have been recorded. One exception is the incomplete extinction of secretion of albumin which continues at a reduced rate in hepatoma x fibroblast hybrids while two other characteristic liver functions [production of tyrosine aminotransferase (EC 2.6.1.5) and its inducibility by corticosteroids, production of aldolase B(EC 4.1.2.7)] undergo total extinction†.

Although doubts about the significance of extinction have been expressed (see ref. 2), the view that this phenomenon reflects a mechanism of regulation of gene activity in normal differentiation is supported by the second finding, namely that of re-expression of luxury functions by hybrid cells of the described type upon loss of the chromosomes presumably responsible for extinction. A first observation on the re-expression of a luxury function was recorded by Klebe et al. (3) on hybrids between cells of a mouse renal adeno-carcinoma (RAG, characterized by the production of large amounts of an eserine-insensitive esterase, ES-2) and human diploid fibroblasts. In the hybrid cells that retained many human chromosomes, ES-2 was absent and further loss of human chromosomes led to re-expression of ES-2. Moreover, the karyotypic characteristics of the parental cells of these hybrids enabled Klebe et al. (3) to establish a correlation between loss of definite human chromosomes (probably C6) and re-expression of ES-2. Similar observations have been made in our Laboratory on two luxury functions of hepatoma cells: inducibility of tyrosine aminotransferase and production of aldolase B (4, 5).

Taken together, these observations suggest that: (a) in hybrids of the type described, the maintenance of extinction of a given luxury function requires the continuous presence of definite chromosomes of the parent not expressing this function; (b) the "epigenotype" of a differentiated cell is endowed with a high degree of stability since it can be propagated over many cell generations without being expressed; (c) the sites of stable epigenetic changes characteristic of different cell types are either in the chromosomes themselves or, if they are elsewhere in the cell, they are relayed to the chromosomes in order to become effective.

Because of the nature of the luxury functions investigated and of the generally low frequency of their re-expression (which depended on the spontaneous and apparently random loss of the chromosomes), in all three cases referred to above the occurrence of re-expression required the application of laborious (biochemical or physicochemical) assays to numerous "segregated" hybrid clones. We have tried both to increase the frequency of re-expression and to facilitate its detection by using (f) the technique of Pontecorvo (6) for

Abbreviations: A, acrocentric chromosomes; B, bi-armed chromosomes; Cl., clone.

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† A complete review of the relevant literature will be found in ref. 2.
the induction of directed chromosome losses (x-irradiation of one of the parental populations prior to hybridization), (ii) the cross melanoma cells x irradiated fibroblasts, because the re-expression of melanogenesis can be detected simply by visual inspection so that large hybrid populations can be screened. Melanoma x fibroblast hybrids were thought to present the additional advantage of having a rather complete and stable karyotype (7) to which (a) the two parents contribute a practically equal (50 and 51) total number of chromosomes and (b) the great majority of acrocentric (A) chromosomes (43 out of 101) are contributed by the mouse parent and the great majority (42) of bi-armed chromosomes (B) are contributed by the hamster parent, so that a marked preferential loss of mouse chromosomes should be correlated with a decrease, in the hybrids, of the proportion of acrocentric chromosomes (A/B ratio).

As will be seen below, the success of our limited attempts to induce the directed loss of mouse chromosomes remains uncertain, but the experiments led to some unexpected observations on the dependence of extinction and/or re-expression on gene dosage which appear to us of sufficient interest to justify a preliminary account at this time. A detailed report will be published in due time.

MATERIALS AND METHODS

Three cell lines have been used: (i) 3460-3-B, a recently isolated subclone of the pigmented 8-Azaguanine-resistant Syrian hamster melanoma 3460-3 (1); (ii) 3460-3, Cl. 4-6 (hereafter referred to as Cl. 4-6), a subclone of 2x cells isolated from 3460-3 in this laboratory; the cells of Cl.4-6 are more pigmented than those of 3460-3-B; (iii) LM (TK-) Cl. 1D (hereafter referred to as Cl. 1D), a clone of BrdU-resistant mouse fibroblasts (8).

Hybrids were selected and maintained in Dulbecco’s modification of Eagle’s minimal essential medium supplemented with 10% fetal calf serum with the addition of 1 × 10^-4 M hyposaxanthine, 4 × 10^-7 M aminopterin and 1.6 × 10^-4 M thymidine (9).

For hybridization, mixed suspensions of Cl. 1D fibroblasts and melanoma cells were exposed to UV-inactivated Sendai virus as described by Harris and Watkins (10). Immediately thereafter, the cell suspension was diluted with the selective medium described above and distributed in a large number of 10-cm Falcon plastic petri dishes. The latter were then incubated at 37°C in a humidified atmosphere of 7% CO2 and 93% air.

As soon as colonies of hybrid cells became distinctly visible, porcelain cylinders rimmed with silicone grease were placed around some of them. These colonies were examined frequently and, when sufficiently large, trypsinized, and transferred to petri dishes for karyological examination and/or further subcultivation.

Irradiation (unfiltered x-rays: 50 kV, 15 mA, dose rate 240 R/min) was performed on trypsinized Cl. 1D fibroblasts resuspended in growth medium.

RESULTS

Hybridization of melanoma cells with irradiated fibroblasts

Two exploratory experiments were performed that differed in the x-ray dose given to the Cl. 1D fibroblasts [400 R (8% survival) and 700 R (1.2% survival), respectively] immediately prior to fusion with 1x melanoma cells of line 3460-3-B. Within a few days, the presence of numerous colonies of highly polymorphic unpigmented hybrid cells was observed. Many of these colonies eventually degenerated. Early karyological examination of a few metaphases from several rapidly growing, fibroblast-like colonies formed in each of the experiments showed that (i) they must have contained, to begin with, one genome of each parent but, after about 20 generations, had lost more chromosomes than the 3460-3 x Cl. 1D hybrids described (7); (ii) by comparison with the expected mean initial number of chromosomes, the average loss of chromosomes was somewhat more pronounced in hybrids of the second experiment (16%) than in those of the first one (9%) and the ranges of A and B chromosomes were increased in both experiments; (iii) only in one out of four clones from each experiment, the A/B ratio had decreased from the expected value of 1.0-0.83 and 0.9, respectively, suggesting a greater loss of chromosomes of the irradiated parent; in all of the other clones, the deviation from the expected ratio was in the opposite direction (average A/B = 1.3).

Thus, no systematic preferential loss of chromosomes of the irradiated parent was detected in our experiments (however, see Discussion) and they are described at this time only because protracted observation of the dishes revealed, in each of the experiments, the greatly delayed formation of a very slowly growing colony of large peculiar looking cells, the study of which proved to be instructive.

In the first experiment, colony 8B was initially composed of unpigmented cells. After transfer from the cylinder to a Petri dish, it gave rise to a dense multilayer of unpigmented fibroblast-like cells within which the late appearance of several (independent?) foci of very darkly pigmented cells of clearly different, epithelial morphology was noticed. The isolation of some of these foci led to the establishment of culture 8Bβ which, to begin with, appeared to comprise uniquely (or an overwhelming majority of) melanin “over-producing” epithelial cells. Karyological analysis of 8Bβ cells performed at this time proved them to be 2x melanoma x 1x fibroblast hybrids which, by comparison with the expected chromosome number (Table 1), had lost about 11% of the chromosomes without significant modification of the expected A/B ratio (0.64 for this type of hybrid).

| Table 1. Karyotypes of parental and hybrid cells |
|--------------------|----------------|-----|-----|-----|
| Cell type* | Mean number of chromosomes |
| | Total | A | B | A/B |
| Cl1D (21) | 51 (46-54) | 43 | 8 | 5.38 |
| Cl460-3-B (20) | 50 (49-51) | 8 | 42 | 0.19 |
| Expected Cl1D x Cl460-3-B (2a) | 151 (144-156) | 59 | 92 | 0.64 |
| 8Bβ (18) | 134 (120-140) | 53 | 81 | 0.65 |
| G7 (20) | 129 (118-137) | 51 | 78 | 0.65 |
| G7B (10) | 129 (124-132) | 50 | 79 | 0.63 |
| G7E (24) | 127 (114-132) | 48 | 79 | 0.61 |
| G7F (29) | 127 (118-133) | 46 | 81 | 0.57 |
| G7H (16) | 128 (123-134) | 50 | 78 | 0.64 |
| 11c’s (14) | 122 (112-128) | 42 | 80 | 0.53 |
| 11c’sU (9) | 113 (110-117) | 39 | 74 | 0.53 |

* In parenthesis is the number of metaphases examined. 
† In parenthesis is the observed range.
On continued subcultivation, the 8Bβ population appeared to contain an increasing proportion of unpigmented fibroblast-like cells whose relationship with the darkly pigmented cells remains uncertain, since the very late isolation of 8Bβ by "scraping" of the foci did not guarantee the isolation of a pure population. An attempt at cloning 8Bβ at this time "by hand" (isolation of single cells with the help of a micropipette; under a binocular, and inoculation of each of them into a well of a Falcon Microtest II tissue culture plate) resulted in a single success; a single cell, whose pigmentation could unfortunately not be ascertained, gave rise to a colony (G7) comprising predominantly unpigmented cells but also a few pigmented ones. G7 was transferred to a culture bottle; it gave rise, like 8Bβ, to a culture of unpigmented fibroblast-like cells with "islands" of very darkly pigmented cells of distinctly different (epithelial-like) morphology (Fig. 1). Recloning of this culture in the usual way resulted in the isolation of four subclones, G7B, G7E, G7F, and G7H, of which G7B was composed of unpigmented fibroblast-like cells and G7E, G7F, and G7H were composed of very darkly pigmented epithelial cells (Figs. 2 and 3); all of these subclones remained stable with respect to phenotype on further subculture (thus far, about 20 generations). Since in this case we are surely dealing with subclones of a clone (G7), it is clear that a single hybrid cell of the 2s melanoma x 1s fibroblast type can give rise to both pigmented and unpigmented progeny. However, the derivation of pigmented and unpigmented cells from each other has in no case been established.

Moreover, early karyological examination of G7 and of the four subclones (Table 1) revealed no statistically significant differences between them: compared with 8Bβ, they all had lost from 5 to 7 additional chromosomes, and their A/B ratios (0.6–0.65) were very near to the expected 0.64 value (except in the case of subclone G7F: A/B = 0.57), indicating that the frequency of loss of acrocentric chromosomes was equal to, or slightly superior to that of bi-armed chromosomes (it must be kept in mind that eight of the bi-armed chromosomes, on the average, are of Cl. 1D origin). Thus, no correlation between karyotype and phenotype of the different clones was detected.

Very similar observations were made in the second experiment. The greatly delayed formation of a colony (11c), initially composed of large round unpigmented cells was observed, within which the presence of a few very darkly pigmented cells was noticed three days later. The cells were trypsinized and re-inoculated into a Petri dish wherein they gave rise to a population of predominantly unpigmented cells (11c') with some foci of very darkly pigmented cells. (Detached clusters of such cells were also seen floating in the medium in a typical "melanoma-like" fashion). Some of the pigmented foci were scraped with, and drawn into a micropipette. Upon re-inoculation, a culture (11c'a) of mostly very darkly pigmented cells was obtained, from which an unpigmented clone (11c'aU) was soon isolated.

The results of the early karyological examination of 11c'a, and 11c'aU cells proved them also to be hybrids of the 3460-3-B (2e) x Cl. 1D (1s) type which, by comparison with the expected hybrids of this constitution (Table 1), had undergone, respectively, losses of 19 and 25% of the total number of chromosomes and had lost more acrocentric than bi-armed chromosomes with the consequent decrease

**Figs. 1–3. Microphotographs of cells of (1) Culture G7, (2) Cl. G7B, and (3) Cl. G7F.**
of the A/B ratios to 0.53. More importantly, it is to be noticed that here, like in the preceding experiments, the A/B ratios were found to be similar in the pigmented population 11c′a and in the unpigmented clone 11c′aU derived from it. We shall return to the significance of this fact in the Discussion.

Since the derivation of pigmented and unpigmented hybrids from each other could not be unequivocally established either in this case or in the case of the clonal derivatives of 8B9 in Exp. 1, it is not possible to state at this time whether, in the pigment producing hybrids, there is lack of extinction or re-expression (correlated with loss of a few specific but unidentified fibroblast chromosomes) of pigment synthesis. The only obvious conclusion from these observations is that there is a relation between the dosage of melanoma genes and one of these phenomena since pigmented 1s melanoma x fibroblast hybrids have never been observed.

Crosses of 2s melanoma cells with unirradiated fibroblasts

In order to confirm the conclusion arrived at above and, hopefully, to elucidate the significance of the recorded observations, crosses were undertaken between cells of the (2s) melanoma 4-6§ and unirradiated Cl. 1D fibroblasts. Early observation of the fusion dishes permitted the detection of hybrid colonies at the 4-8 cell stage. At this time already three types of hybrid colonies could be distinguished, composed respectively (i) uniquely of definitely more darkly pigmented cells than those of the parental line 4-6 and of epithelial morphology, (ii) uniquely of unpigmented cells, and (iii) of both pigmented and unpigmented cells ("phenotypic mosaics"). Three colonies of type 1, eight of type 2, and 13 of type 3 were isolated and periodically examined during the following month. These observations showed that two colonies of type 1, and six of type 2 retained the initial phenotype, while the remainder became "mosaics." In subcultures of the latter, as well as in the 13 colonies of type 3, one of the cell types (pigmented or unirradiated) gradually became vastly predominant, the evolution (selection?) of the population in either direction occurring with roughly equal frequency.

Karyological analysis of 16 of these hybrids performed at this stage showed that their phenotype instability is paralleled by inordinate karyological variability. Whatever their phenotype when they were first detected or that at the moment of the karyological examination (about 1 month after their isolation), the modal and mean total chromosome numbers in the different clones cover a wide range of values from nearly the expected mean of 147 down to 100, with a very large intrachromosomal variation (and sometimes bimodal distribution). The A/B ratios were also found to be extremely variable in the different clones and subpopulations, but decrease from the expected 0.65 value was observed only in two cases out of 19; A/B ratios of 0.65-0.70 were observed in four cases and of 0.71-0.88 in the remaining 13 cases. Clearly, in these hybrids, there is spontaneous preferential loss of bi-armed chromosomes.

Owing to this variability (which indicates continuing chromosome losses), no correlation could be established thus far between karyotype and phenotype of these hybrids.

‡ The mean numbers of chromosomes in Cl. 4-6 are: total, 96; acrocentric, 16; bi-armed, 80.

DISCUSSION

In the light of these observations, we may now reconsider some of the questions left open above.

The first of these questions is whether a preferential loss of mouse chromosomes was obtained in the irradiation experiments. It will be recalled that 8Bβ cells did not have a clearly modified A/B ratio. Taken by itself, this fact suggests that 8Bβ arose from the fusion of a Cl. 1D fibroblast with a 2s melanoma cell, some such cells being always present in cultures of 3460-3-B. On the other hand, the decreased A/B ratio in the 11c′a and 11c′aU cells suggest that 11c′ owes its origin to the effect observed by Pontecorvo (6) for supra-optimal irradiation: loss of chromosomes of the irradiated parent, accompanied by doubling up of the genome of the unirradiated one. However, we have seen above that 2s melanoma x Cl. 1D hybrids tend to lose preferentially bi-armed chromosomes and generally have increased A/B ratios. Consideration of this fact leads to the conclusion that, unless there is a difference in this respect between hybrids resulting from crosses of Cl. 1D with 3460-3-B and 4-6, this tendency of 2s melanoma hybrids may have obscured the successful induction of loss of mouse chromosomes in 8Bβ and 11c′ cells; and that our failure to observe the induced loss of fibroblast chromosomes in the 1s melanoma hybrids may have been due to premature karyological analysis (before completion of chromosome losses).

The second question to be discussed is: was the formation of pigmented hybrids due to lack of extinction or to re-expression of melanogenesis? This question was not solved by the study of pigmented hybrids obtained in the irradiation experiments even though the late appearance of pigmented cells in clone G7 favored the re-expression mechanism.

Very late appearance of pigmented cells was observed also in several of the initially unpigmented hybrids of the 2s melanoma cross and leaves but little doubt that we are dealing with re-expression. However, the evolution of one hybrid colony of type 1 formed in this cross makes it probable that the reverse change occurs also.

Turning to the formation of both pigmented and unpigmented hybrids in the same 2s melanoma x Cl. 1D cross, it is clear that it may be (a) correlated with the retention versus early loss of some specific, unidentified fibroblast chromosomes producing the hypothetical substance responsible for extinction of melanogenesis, or (b) due to the effect of the relative dosage of fibroblastic regulatory genes and melanoma genes involved in pigment production. The choice between these two (not mutually exclusive) hypotheses must await the detailed karyological analysis of the two types of (still segregating) hybrids. We are encouraged by the frequent similarity of the chromosome numbers and A/B ratios in pigmented and unpigmented hybrids, which indicates that the fibroblast genes responsible for extinction cannot be numerous.

Another problem posed by our observations is the mechanism of the obvious overproduction of melanin by some of the described hybrids, for it makes one wonder whether it is not due to the induction of melanin production by the fibroblast genome. Induction of mouse serum albumin synthesis by fibroblast genomes has been observed in 2s hepatoma x fibroblast hybrids (11) (this is the third important finding mentioned in the Introduction).

Lastly, we wish to remark that there is at least a formal analogy between the phenomenon of extinction and re-expression on the one hand and, on the other, those of (a) suppression
or decrease of malignancy in hybrids between highly malignant and non- (or less) malignant cells; (b) the recovery of malignancy by segregated hybrids that have lost a certain number of (definite?) "suppressing" chromosomes (12). Further, there is suggestive evidence that, like extinction of luxury functions, suppression or reduction of malignancy depends on the relative dosage of chromosomes (or genes) of the malignant and the nonmalignant parent (13). Finally, it has been shown that cells transformed by oncogenic viruses that lost contact inhibition, can give rise to variants that have recovered contact inhibition; this is accompanied by increase of chromosome number. Re-revertants lacking contact inhibition can be selected from these variants. It appears that re-reversion is correlated with a decrease of chromosome number (14, 15). Thus, gene dosage apparently affects the transformed state, as judged in particular by the criterion of contact inhibition. Identification of the individual chromosomes, the relative proportions of which are responsible for these variations is underway (16). These observations raise again the question whether malignancy and/or transformation, are based, like the changes observed in our experiments, on epigenetic changes rather than on truly genetic ones.

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