Epigenetic activation of phenylalanine hydroxylase in mouse erythroleukemia cells by the cytoplast of rat hepatoma cells (enucleation/cell fusion/cytoplasmic control)

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ABSTRACT Friend mouse erythroleukemia cells do not synthesize detectable levels of phenylalanine hydroxylase [phenylalanine 4-monoxygenase; L-phenylalanine, tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] and hence are unable to grow in medium totally lacking tyrosine. These cells were fused with the cytoplasts of rat hepatoma cells that synthesize phenylalanine hydroxylase constitutively. Cytoplastic hybrids [cybrids, Bunn, C. L., Douglas, C. W. & Eisenstadt, J. M. (1974) Proc. Natl. Acad. Sci. USA 71, 1681–1685] were selected in medium without tyrosine. Cybrid clones expressed phenylalanine hydroxylase enzyme, which was of mouse type as determined by immunotitration and isoelectric focusing. This phenotype has been maintained even in the absence of any selective pressure. In contrast, in whole cell hybrids derived between the same parents, the expression of the phenylalanine hydroxylase gene was totally extinguished. One interpretation of these results is that the cytoplast of rat hepatoma cells contains a positively acting factor(s) for the phenylalanine hydroxylase gene that brings about the activation of this gene in erythroleukemia cells.

The study of the expression of differentiated functions in somatic cell hybrids that have been derived from cells expressing varied functions has been used extensively for analyzing the regulatory mechanisms involved in the expression of these functions (see refs. 1–3 for reviews). The role of the egg cytoplasm in directing the determinative steps of the fertilized nucleus is well established (4–7). Recently, it has been shown by De Robertis and Gurdon that, when a somatic cell nucleus is introduced into an oocyte, the oocyte cytoplasm brings about both activation and repression of different genes in the newly introduced nucleus (8). These experiments suggest the possible existence of both positive and negative regulatory elements in the egg cytoplasm that might determine the ontogeny of various cell types present in the animal. We have attempted to use cultured cells that express functions characteristic of the tissues from which they have been derived to search for cytoplasmic regulatory factors that influence nuclear gene expression. In an earlier report (9), we demonstrated that the cytoplast of nonerythroid cells, when introduced into Friend mouse erythroleukemia (MEL) cells, can bring about the permanent extinction of hemoglobin induction in these cells. These experiments suggested that a self-perpetuating negative regulatory element(s) exists in the cytoplast.

The present report describes evidence for the possible existence of positive cytoplasmic regulatory factors that are also self-perpetuating in nature and act on nuclear genes to bring about the activation of a normally silent gene. The system we have chosen is the activation of phenylalanine hydroxylase in [phenylalanine 4-monoxygenase; L-phenylalanine, tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] in MEL cell cybrids derived by fusing MEL cells with the cytoplast of rat hepatoma cells. These latter cells express phenylalanine hydroxylase constitutively and hence are able to grow in the absence of exogenous tyrosine (10, 11). MEL cells, which have no detectable level of phenylalanine hydroxylase, cannot survive in medium lacking tyrosine. Thus, this system offers the advantage of a built-in selective system in looking for a regulatory factor.

MATERIALS AND METHODS

Cells. FT-2 is a clonal line derived from rat hepatoma cell line FU-5-5 (12) by cloning in Tyr− medium (regular growth medium lacking tyrosine and supplemented with 2 times concentrated phenylalanine and 10% dialyzed fetal calf serum [GIBCO]). This cell line is also resistant to 6-thioguanine and ouabain and grows with the same rate in medium either with or without tyrosine. C19TK is a clonal line of MEL 745 cells, which grows in suspension and is resistant to 0.1 mM BrdUrd; it was kindly provided by R. Rifkind. The rat glioma cell line C6 was obtained from American Type Culture Collection (CCL 107). All the cells were grown in medium W10 (modified improved minimum essential medium supplemented with 10% fetal calf serum) (13).

Enucleation and Cell Fusion. FT-2 cells were enucleated on concanavalin A-coated plastic coverslips according to the method of Gopalakrishnan and Thompson (14) with the slight modification that centrifugation was at 14,000 rpm for 50 min at 30°C. When enucleating C6 cells, we added 1 μg of Colcemid per ml to the enucleation medium. The enucleated cells on concanavalin A-coated coverslips were fused with MEL cells as described (9). After fusion, the coverslips were washed twice with W10 medium and placed in 100-mm dishes in W10 with penicillin, streptomycin, and 2.5 μg of fungizone (GIBCO) per ml. After 3 days, the cells were harvested, washed once in Tyr− medium by centrifugation, and plated in separate 100-mm dishes at different concentrations (105, 104, and 103 cells per ml) in Tyr− BrdUrd medium (Tyr− medium supplemented with 0.1-mM BrdUrd) to exclude any residual intact FT-2 cells as well as any whole cell hybrids. The medium was changed by collecting the cells by centrifugation and plating them back in the same dishes. When the cybrid populations began active growth (usually 4–6 weeks after fusion), they were maintained in Tyr− medium. Whole cell hybrids between FT-3 and C19TK cells were obtained by fusing 1 X 106 cells of each parent with approximately 500 hemagglutinating units of β-propiolac-
tissue-inactivated Sendai virus as described (9). After fusion, the cells were plated in W10 medium in 100-mm dishes. The medium was replaced by W10 HAT medium containing 0.1-mM hypoxanthine, 1 μM aminopterin, 16 μM thymidine, and 0.8 mM glycine the following day (15). Care was taken to save and replicate all floating cells while changing the medium.

Chromosome Analysis. The cells were treated with 0.5 μg of Colcemid per ml for 3–4 hr followed by 15–20 min of swelling in 75 mM KCl at 37°C. The cell suspension was then fixed in methanol/acetic acid (3:1) and spreads were made on glass slides. They were then stained with Giemsa and photographed (at least 15 spreads for each cell line).

Phenylalanine Hydroxylase Assay. Tissue culture cells for enzyme assay were harvested at confluence, washed once in ice-cold phosphate-buffered saline, and stored at −80°C until use. The cell pellets were suspended in 5 vol of 0.1 M potassium phosphate buffer (pH 6.8) and lysed either by freezing and thawing twice in an aceton/dry ice bath and 37°C water bath, or by sonication. The lysates were centrifuged at 5°C in an Eppendorf microcentrifuge for 15 min, and the supernatants were used for the enzyme assay. ACI rat liver and DBA/2 mouse liver extracts were prepared by homogenization of the liver in 5 vol of 0.1 M potassium phosphate buffer (pH 6.8) with a motor driven Teflon homogenizer at 5°C. The homogenates were centrifuged in a Sorvall RC-2B centrifuge at 10,000 x g for 15 min. The supernatants were dispensed into small volumes and kept frozen at −80°C until used for enzyme assay. The phenylalanine hydroxylase assay was performed according to a modification of the procedure of Guroff and Abramowitz (16). The assay was carried out in a total volume of 0.08 ml by using DL-6-methyl-5,6,7,8-tetrahydropterine as cofactor, which was kept reduced in an atmosphere of hydrogen by using platinum oxide catalyst. The assay mixture contained: L-phenylalanine at 0.625 mM; dithiothreitol at 14.25 mM; NADH at 10.5 mM; potassium phosphate (pH 6.8) at 162.5 mM; catalase at 400 units; cofactor at 0.5 mM. The specific activity of the enzyme is expressed as nanomoles of L-tyrosine formed per min/mg of protein at 30°C. Protein estimation was carried out according to Lowry’s method with bovine serum albumin as standard.

Immunotitration. Cell extracts were diluted so that each had the same concentration of enzyme. Portions (30 μl) of different dilutions of the IgG fraction of an antiserum prepared against purified rat liver phenylalanine hydroxylase, kindly provided by S. Milstein and S. Kaufman, were added to 30 μl of each different cell extract. After incubation at 5°C for 2–3 hr, the extracts were centrifuged for 10 min in an Eppendorf microcentrifuge. Aliquots (20 μl) of the supernatant were assayed in duplicate for phenylalanine hydroxylase. The enzyme activity without an antibody is taken as 100%.

Isoelectric Focusing. Phenylalanine hydroxylase fractions, obtained by (NH₄)₂SO₄ precipitation (17) from extracts of ACI rat liver, DBA/2 mouse liver, FT-2, and one of the cybrids, were focused in a 5% polyacrylamide gel crosslinked with bis-acrylamylacrylamide (18). The gel also contained 1% (vol/vol) Bio-Lyte 4/6 and 2 parts Bio-Lyte 3/10. Isoelectric focusing of the extracts was carried out in a LKB multiphor apparatus at 5°C for 4 hr at 1300 V. After electrophoresis, 0.25-cm slices were transferred to assay tubes containing 50 μl of 0.14 M diithiothreitol solution and the assay was carried out at 30°C as described. The reaction was stopped by the addition of 50 μl of 1.2 M sodium acetate (pH 5.5) followed by the addition of 100 μl of 1.12 M N-ethylmaleimide to neutralize excess diithiothreitol, which otherwise interfered with the iodination step in the assay (16).

RESULTS

Properties of Cybrids Formed between Eueucleated Rat Hepatoma FT-2 Cells and MEL Cells. When C19TK MEL cells were plated in Tyr− medium at a density of 10⁶ cells/ml, they divided only once. By 2 weeks, most of the cells had died. However, there remained a few viable cells even after 2 months, but no growth was ever observed. We took advantage of this property to test for the possible presence of a cytoplasmic regulatory factor(s) for phenylalanine hydroxylase in hepatoma cells, which, when introduced into MEL cells, might activate the normally silent phenylalanine hydroxylase gene of the MEL cells and thereby allow vigorous growth in Tyr− medium.

Rat hepatoma cells FT-2 synthesize a high level of phenylalanine hydroxylase constitutively and hence are able to grow very well in Tyr− medium, whereas C19TK cells have no detectable level of enzyme activity and do not survive in Tyr− medium. Cybrid populations isolated from four independent cybridization experiments between FT-2 and C19TK grew continuously in Tyr− medium. The properties of the cybrids are given in Table 1. The cybrids possessed about 25% of the enzyme activity found in rat hepatoma cells. The cybrids appeared with a frequency of 10⁻⁵ to 10⁻⁶. Subclones isolated from cybrids possessed enzyme activity similar to the uncloned cybrid populations. The cybrid PHC-3 was maintained under nonselective conditions and the enzyme activity was measured every week. There was no detectable change in the specific activity of the enzyme for up to 1 month (data not shown). These results suggest that, once the enzyme is activated in MEL cells by the hepatoma cytoplasm, it is stably maintained. The lactate dehydrogenase isozyme expressed in the cybrids was identical to the parental MEL cells (Fig. 1), and all the cybrids had the same chromosome complement as the parental MEL cells (Table 1).

Control experiments established: (i) cybrids derived from L cell cytoplasts and MEL cells by using the cytoplasmic marker of chloramphenicol resistance (9) did not grow when plated in Tyr− medium (L cells do not grow in Tyr− medium and possess no detectable level of phenylalanine hydroxylase); (ii) no cybrids were obtained when rat glioma cytoplasts (these cells do not grow in Tyr− medium) were fused with C19TK cells and then selected in Tyr− medium; and (iii) no growth of C19TK cells occurred in Tyr− medium, after an initial doubling, for up to 6 months. These experiments suggest that the continuous growth of MEL cells in Tyr− medium after fusion with hepatoma cytoplasts is not due either to the spontaneous activation of the phenylalanine hydroxylase gene of MEL cells or to the fusion event per se.

Whole Cell Hybrids between FT-2 and MEL Cells. Hybrids between FT-2 and C19TK cells were obtained as described in Materials and Methods. The properties of five independently isolated hybrids are given in Table 1. The hybrid nature of these cells was established by chromosomal analysis as well as by lactate dehydrogenase isozyme analysis (Table 1 and Fig. 1). All of these hybrids grew in suspension and were inducible for hemoglobin (Table 1). (No hybrids that grew attached to the substrate were obtained from the fusion of FT-2 with C19TK.) When the hybrids were analyzed for phenylalanine hydroxylase activity about 1 month after isolation, none had a detectable level of activity (Table 1). Moreover, hybrids selected in HAT medium did not grow when plated in Tyr− medium.

Characterization of Phenylalanine Hydroxylase in Cybrids. If the enzyme expressed in MEL cells is due to the activation of the normally silent MEL gene for phenylalanine hydroxylase, then the expressed enzyme should be of mouse type because the cytoplast donor was a rat cell line. Fig. 2 shows the
inactivation of phenylalanine hydroxylase from rat liver, mouse liver, rat parental FT-2, and cybrid PHC-3 extracts by the IgG fraction of antiserum against purified rat liver phenylalanine hydroxylase. The slopes of the inactivation curve for rat liver enzyme and FT-2 extracts were similar, whereas the curves for mouse liver enzyme and cybrid PHC-3 extract were similar.

Analyses of another cybrid (PHC-2) gave the same result. These data suggest that the enzyme expressed in the cybrids is of the mouse type. A similar conclusion was arrived at based on immuno diffusion studies (data not shown).

Additional evidence for the identity of the cybrid and mouse liver enzyme was obtained by isoelectric focusing. Fig. 3 shows that the migration of cybrid PHC-3 enzyme was similar to that of the mouse liver enzyme, but different from that of the rat liver and the rat hepatoma enzymes. Thus, phenylalanine hydroxylase in MEL cybrids appears to be due to activation of the normally silent phenylalanine hydroxylase gene in the recipient cell genome.

Production of Second Generation Cybrids. We have succeeded in constructing "second generation" cybrids by fusing the cytoplasm of PHC-3 with a 6-thioguanine-resistant MEL cell line GM86TG (9) followed by selection in Tyr⁻ medium supplemented with 0.1 mM 6-thioguanine to exclude any residual intact PHC-3 cells (data now shown). Independently isolated cybrid populations, PHC-1 and PHC-2, had high levels of phenylalanine hydroxylase activity, whereas GM86TG had no detectable phenylalanine hydroxylase activity. The activation of phenylalanine hydroxylase in MEL cells appears, therefore, to be due to a "continuous action" mechanism, perhaps by a self-perpetuating regulatory element.

DISCUSSION

We have shown that the fusion of rat hepatoma cytoplasts with MEL cells results in the activation of the phenylalanine hydroxylase gene of MEL cells. Our data suggest that the rat hepatoma cytoplasm contains a self-perpetuating positive
These results paper deal with the been studied reported (23). In expression several other study mouse in the cells with a number of (19, 2). Factor(s) responsible regulatory element(s) for the phenylalanine hydroxylase gene is perpetuated through many generations.

An unexpected aspect of the present study is that the expression of the phenylalanine hydroxylase gene in whole cell hybrids formed between FT-2 and MEL cells is totally extinguished, whereas the same function is activated in MEL cells by the introduction of the hepatoma cytoplasm. If the rat hepatoma cytoplasm carries a positive regulator(s) for the phenylalanine hydroxylase gene, then even in whole cell hybrids the factor(s) should be present and operative. A similar result has been obtained with hybrids formed between L cells and FT-2 cells (unpublished results). One explanation for this paradox could be the aneuploid nature of the rat hepatoma cells. Exposure of the rat hepatoma genome to the nonhepatic cell content could result in the production of a negative regulator for the phenylalanine hydroxylase gene that may be responsible for the total extinction of the phenylalanine hydroxylase gene from both the parental cell genomes. This could have arisen from an imbalance in the chromosomes coding for the positive and negative regulatory elements for the phenylalanine hydroxylase gene in the aneuploid rat hepatoma cell compared to the "near diploid" MEL cells. One could envisage other mechanisms to explain this paradoxical behavior of the phenylalanine hydroxylase gene in whole cell hybrids.

Transfer of a negative regulatory factor(s) from fibroblasts that permanently inhibits globin gene induction in MEL cybrids. Regulatory factor(s) responsible for the activation of the normally silent MEL phenylalanine hydroxylase gene.

Activation of differentiated functions in hybrids formed between two different differentiated cells has been studied in a number of systems. It was shown first by Weiss and coworkers (19, 20) that the fusion of albumin-synthesizing rat hepatoma cells with nonhepatic mouse cells can result in the activation of the mouse albumin gene. Similar results have been obtained in the activation of the human albumin gene in hybrids formed between mouse hepatoma cells and human cells (21, 22). The study of the activation phenomenon has also been extended to several other systems (1–3). Activation of fibroblast globin gene expression in MEL–human fibroblast hybrids has recently been reported (23). Thus far, the activation phenomenon has only been studied in whole cell hybrids. The results presented in this paper deal with the activation of a hepatic function (phenylalanine hydroxylase) in cybrids rather than whole cell hybrids. These results suggest that the nuclear gene activity for a differentiated function is mediated by cytoplasmic regulatory elements. Furthermore, the activation of phenylalanine hydroxylase in the "second generation" cybrids suggests that the regulatory element(s) for the phenylalanine hydroxylase gene is perpetuated through many generations.

FIG. 2. Immunotitration of rat liver (○), FT-2 (■), mouse liver (○), and PHC-3 (○) extracts with the IgG fraction of antiserum to rat liver phenylalanine hydroxylase. The immunotitration was carried out as described in Materials and Methods. The amount of enzyme used (in milliliters) of different extracts was: rat liver, 30, FT-2, 35.3; mouse liver, 39.4; PHC-3, 32.6. One milliliter of phenylalanine hydroxylase is defined as 1 nmol of L-tyrosine formed per min at 30°C.

FIG. 3. Ioselectric focusing of rat liver (○), mouse liver (○), rat parental FT-2 (■), and cybrid PHC-3 (○) extracts. Ioselectric focusing and the enzyme assay in the gel slices were carried out as described in Materials and Methods. The amount of enzyme (in milliliters) loaded was: rat liver, 5.6; mouse liver, 4.2; FT-2, 0.6; PHC-3, 0.48. The extracts were loaded between 19 and 19.5 cm from the anode. The pH (1A) of the gel was measured by using a flat surface electrode at the end of electrophoresis.

Regulatory factor(s) responsible for the activation of the normally silent MEL phenylalanine hydroxylase gene.
has been reported (9). The extinction of hemoglobin induction in cybrids occurred only in a particular isolate of MEL cells (Ikawa’s line T3c1-2 and its derivatives [24]). MEL cells used in the present studies (C19TK) did not show this phenomenon of extinction when fused with cytoplasts of nonerythroid cells (9). The extinction and activation of phenylalanine hydroxylase in whole cell hybrids and cybrids, respectively, might offer a distinct advantage for studying the mechanisms of negative and positive regulation in the same system. It would be of interest to know whether the extinction of other hepatic functions in whole cell hybrids formed between a hepatoma cell line and nonhepatic cells (25–27) would behave in a similar way to phenylalanine hydroxylase. Other hepatic functions might also be activated in cybrids in contrast to their extinction in hybrids.

There are numerous examples in developmental biology for the cytoplasmic control of nuclear gene activity (28). The present system is an example of permanent activation of a specific gene by cytoplasmic factors in cultured cells. Because of the advantages of the built-in selective system, activation of the phenylalanine hydroxylase gene offers itself as a useful model system for investigating many of the above speculations and the molecular basis of gene activation.

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