Proc. Nat. Acad. Sci. US
Vol. 69, No. 8, pp. 2179–2183, August 1972

Expression of Aryl Hydrocarbon Hydroxylase Induction and Suppression of Tyrosine Aminotransferase Induction in Somatic-Cell Hybrids

(cell fusion/cell culture/enzyme induction)

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Communicated by Julius Axelrod, May 15, 1972

ABSTRACT **Aryl hydrocarbon hydroxylase activity is inducible in mouse 3T3 fibroblasts by benz[α]anthracene, whereas no detectable basal or inducible levels of this enzyme occur in rat-hepatoma tissue culture cells. Conversely, tyrosine aminotransferase activity is inducible in hepatoma cells by dexamethasone, whereas only low noninducible levels of this enzyme exist in 3T3 cells. In hybrids formed by fusion of these two parent lines, levels of inducible hydroxylase activity range from the same as, to more than 20-fold greater than, that in the 3T3 parent; aminotransferase levels remain very low and noninducible in all of these same hybrids. A majority of the 1S-chromosomal complement from each parent is retained in most of these hybrids. The kinetics of hydroxylase induction and degradation, responses of hydroxylase induction to actinomycin D and cycloheximide, and the relative thermolability of the control and induced activities are similar in the 3T3 parent and in the hybrids. Failure to inactivate any of the aminotransferase activity in the hybrids with antibody specific for the rat enzyme indicates that all of the basal noninducible aminotransferase activity is derived from the mouse 3T3 parent.**

Fusion of mammalian cells allows the combination of two genomes that differ in their functional states (1–3). Such hybridization studies may provide a valuable experimental probe for study of the mechanism of genetic expression in mammalian cells. The sequence of events leading to induction of aryl hydrocarbon hydroxylase activity by foreign polycyclic hydrocarbons (4) and the induction of tyrosine aminotransferase (EC 2.6.1.5) activity by corticosteroids (5) have both been studied in detail by the use of cell culture. Hydroxylase activity is an example of the multicomponent, mixed-function oxygenases bound to membranes that require NADPH, NADH, and molecular oxygen for the oxidative metabolism of drugs, insecticides, polycyclic hydrocarbons, and many lipophilic endogenous substrates (4, 6–8). The aminotransferase, on the other hand, is found in the cytosol of several rat-hepatoma cell lines in tissue culture (5). Hydroxylase induction by aromatic hydrocarbons such as BzAnth occurs in mouse 3T3-4(E) cells, whereas there is no detectable enzyme activity in rat-HTC cells. In contrast, aminotransferase induction by corticosteroids, such as dexamethasone, occurs in rat-HTC cells but not in mouse 3T3-4(E) cultures. We show in this report that there are marked differences in the expression of hydroxylase and aminotransferase induction in hybrids between these two parent lines, and suggest that induction of these two enzyme activities is regulated by different mechanisms.

Abbreviations: hydroxylase, aryl hydrocarbon hydroxylase (benzo[α]pyrene hydroxylase); aminotransferase, L-tyrosine: 2-oxoglutarate aminotransferase (EC 2.6.1.5); BzAnth, benz[α]anthracene; dexamethasone, dexamethasone phosphate; and HMT medium, Eagle's culture medium containing 100 μM hypoxanthine, 0.4 μM methotrexate, and 16 μM thymidine.

MATERIALS AND METHODS

The mouse 3T3 fibroblast parent, clone 3T3-4(E), was a generous gift of Dr. Howard Green, Massachusetts, Institute of Technology (9). The AR-1 variant of HTC cells was recently developed (S. L. Levisohn and E. B. Thompson, unpublished data). Sendai virus, obtained from the Research Resources Branch, National Institutes of Arthritis and Metabolic Diseases, was inactivated by β-propiolactone (10). Hypoxanthine was purchased from Aldrich Chemical Co., thymidine from Sigma, and methotrexate from Lederle Pharmaceutical Co. The other chemical reagents, tissue culture materials, and procedures for growth of cells and treatment of cultures with inducers and inhibitors have been described (4, 5, 11).

Cell-Fusion Technique. Clone 3T3-4(E) is deficient in thymidine kinase, and the AR-1 variant line from HTC cells lacks hypoxanthine phosphoribosyltransferase. Thus, fused-cell hybrids that inherit the simultaneous expression of both enzymes are able to grow in HMT medium, whereas neither parent line can survive (3). No *Mycoplasma* contamination of either parent or of any hybrid clone was found. For fusion of the two parent lines, HTC–3T3 ratios of 10:1, 1:1, or 1:10 were used, with a total number of 8–16 × 10^5 cells in 0.25 ml. Each cell mixture was divided and suspended in Dulbecco's isotonic phosphate buffer (pH 7.1); to half of the mixture was added 600, and to the other half 120, hemagglutinating units of β-propiolactone-inactivated Sendai virus (10). After being kept at 0° for 30 min, each group of cell mixtures was concentrated by centrifugation 5 min at 600 × *g*, suspended in Eagle's minimal essential medium at 37°, and seeded in four 60-mm tissue culture dishes. 1 Day later, the Eagle's medium was replaced with HMT medium. In order to assess whether or not "survival" of parent cells in the HMT medium occurred, we also followed unmixed HTC and 3T3 cells through the entire procedure. After 5 weeks of culture in HMT medium, all HTC cells had died, but at each level of virus a few 3T3 cells survived and eventually grew sufficiently to allow comparison with the "hybrid" mass cultures; we refer to the two 3T3 cultures surviving in HMT medium as 3T3 "survivors."

The mass "hybrid" cultures in each of six different petri dishes originally containing various ratios of parental cells and virus were then individually dispersed by trypsin treatment, and plated sparcely enough so that single colonies could be randomly isolated either by the ring method of Puck *et al.* (12) or by recovery of clones growing on bits of broken glass.

The hybrid colonies reported below include examples taken from each petri dish and, therefore, include results of not less than 10 independent hybridization events. The resultant colonies were then grown for 5–15 weeks in HMT medium, and were studied for chromosomal and enzymic content.

Chromosomal Preparations. Chromosomes were prepared as described (13). A minimum of 10 well-spread metaphases for each hybrid, 40 metaphases for the HTC–AR-1 line, and 20 for the 3T3-4(E) parent line was photographed and evaluated.

Enzyme Assays. Both hydroxylase activity and protein concentration were determined in duplicate from the homogenate of 1 to 8 $\times$ 10^6 cells grown in a single 100-mm cell culture dish (4, 14). *One unit* of hydroxylase activity is defined as that amount of enzyme catalyzing per min at 37° the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo[α]pyrene. Hydroxylase-specific activity is expressed as units per mg of cellular protein. Aminotransferase activity was determined with a slight modification of the Diamondstone method (15), and the specific activity is expressed as nmol of *p*-hydroxyphenylpyruvic acid formed per min per mg of cellular protein (5). Protein determinations were based on the Lowry technique (16). Enzyme activities were proportional to the concentration of cellular protein in the assays *in vitro*.

For studies involving hydroxylase induction, all cell lines were grown in Eagle's minimal essential medium with 10% calf serum. At 13 μM BzAnth no toxicity was observed either in a parental line or in any hybrid. Media were changed daily. For studies involving aminotransferase in HTC cells, Swim's medium S-77 with 5% fetal-calf plus 5% calf serum was used; the concentration of inducer was 1 μM. With either enzyme, the rate of induction was similar in either HMT medium or other media. The magnitude of enzyme induction was always studied during logarithmic growth phase, and was determined in two to five separate experiments.

RESULTS

Chromosomal analyses

13 Clones and both parental lines were examined for their chromosomal content. The 3T3 parent contained only telocentric chromosomes (Fig. 1*A*), with a very narrow

TABLE 1. *Relationship between chromosomal complement and maximally inducible hydroxylase and aminotransferase activities in the 3T3 and HTC parent lines, hybrid clones, and two 3T3 "survivor" cultures*

Cell line	Chromosomal mode and range	Bi-armed chromosomes	Estimated number of chromosomes HTC	Estimated number of chromosomes 3T3	Specific hydroxylase activity after BzAnth	Specific amino-transferase activity after dexamethasone
3T3	69 (68–69)	0	(0)	69	3.0*	0.8†
HTC	62 (58–129)	31	62	(0)	<0.02	68
Hybrid 22	157 (152–187)	33	66‡	91‡	62	0.7
Hybrid 23	96 (93–105)	40	80	16	39	1.0
Hybrid 6	104 (91–110)	32	64	40	31	0.9
Hybrid 24	Not done	—	—	—	30	1.2
Hybrid 25	104 (99–113)	28	56	48	25	0.3
Hybrid 12	107 (93–110)	33	66	41	21	2.1
Hybrid 17	118 (114–121)	35	70	48	19	1.0
Hybrid 26	Not done	—	—	—	18	0.9
Hybrid 5	104 (96–115)	33	66	38	18	0.8
Hybrid 19	113 (110–116)	32	64	49	16	1.9
Hybrid 7	112 (105–120)	34	68	44	12	1.2
Hybrid 3	106 (94–111)	32	64	42	12	0.6
Hybrid 29	104 (101–109)	31	62	42	7.0	1.4
Hybrid 11	112 (109–129)	35	70	42	6.8	1.6
Hybrid 28	Not done	—	—	—	5.8	2.6
Hybrid 30	106 (101–107)	34	68	38	2.5	1.2
3T3 "survivor" 1	69 (68–70)	0	(0)	69	2.0	0.8
3T3 "survivor" 2	69 (67–69)	1	(0)	69	2.1	1.0

* Units per mg of cellular homogenate protein. BzAnth or control medium was added to several dishes of cells, and the cultures were harvested, usually after 3, 4, 5, and 6 days, and stored at −20° until assay (4). If cell growth of any clone was poor, the experiment was repeated one or more times to ensure reproducibility. The constitutive hydroxylase specific activity ranged between 0 and 0.10 for 3T3 and between 0.20 and 1.0 for clones 22, 23, 25, and 29 and was not necessarily proportional to the maximally inducible level. In one mass "hybrid" culture exposed to BzAnth, the specific hydroxylase activity ranged between 2.0 and 6.5.

† Nanomol of product formed per min per mg of cellular homogenate protein. For each determination, the cells were grown in a pair of 60-mm tissue culture dishes. One member of each pair was exposed to dexamethasone for 18 hr, while the other served as control; the cells were collected, washed, and assayed (5). Experiments were done simultaneously on cells in logarithmic growth. The constitutive aminotransferase specific activity ranged between 4.0 and 18 for HTC (18 for the induction experiment) and between 0 and 2.6 for the various clones grown in control medium only. In four mass "hybrid" cultures treated with dexamethasone, the aminotransferase specific activity ranged between 1.2 and 1.7.

‡ Approximated by counting the number of bi-armed chromosomes per metaphase and assuming an equal number of telocentric and bi-armed chromosomes derived from the HTC parent, and a random loss of chromosomes in each hybrid.

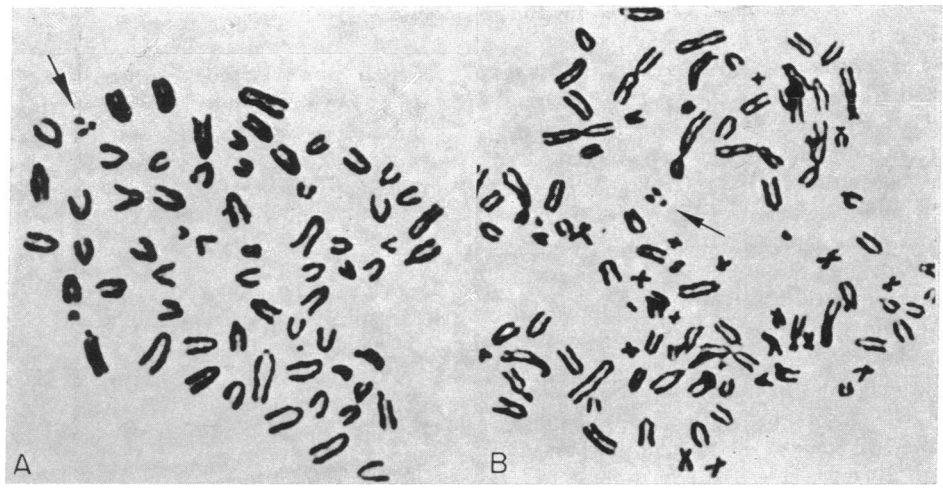

FIG. 1. Photographed metaphases from *A*, the 3T3-4(E) parent; *B*, hybrid 23. *Arrows* indicate the 3T3 marker chromosome.

range. These findings are in accord with the data of Matsuya and Green (9). The HTC parent had a weak mode of 62, with a more variable range of 58–129: about half of the metaphases contained 67 chromosomes or less, and about 25% were about 2S, with 121 or more chromosomes. However, there was always about an equal number of telocentric and bi-armed chromosomes in the HTC parent. A small telocentric marker chromosome with a large unstained region near the contromere could always be identified in the 3T3 parent (Fig. 1*A*), and was found in 10 of the 13 hybrid clones examined. One such hybrid is illustrated in Fig. 1*B*.

Table 1 summarizes the chromosomal complement and extent of enzyme induction in the two parent lines, the somatic-cell hybrids, and the two 3T3 "survivor" mass cultures. Assuming an equal number of telocentric and bi-armed chromosomes originating from the HTC parent, we estimated the chromosomal contribution from each parent by difference. In previous studies involving somatic-cell hybrids (see ref. 17), this same method for estimation in a hybrid of the chromosomal contribution from either parent has been used. Although we realize that such a method may be erroneous by a factor of 10% or more, the fundamental findings presented in this report would still be true. All of the hybrids examined had about a 1S complement of HTC chromosomes; this finding suggests that HTC cells having more than a 1S chromosomal complement either do not fuse, are not viable for the several weeks' growth in HMT medium subsequent to fusion, or develop about a 1S mode after hybridization. In the various hybrids, therefore, we estimate that between 20 and 53 3T3 chromosomes were lost. Possibly even larger numbers of HTC chromosomes have been lost. The general trend in most of the fused hybrids seems to be that a majority of chromosomes from both parents was retained, assuming that most 1S plus 1S hybrids survive. Clone 22 was unique in that about twice as many 3T3 chromosomes were retained, compared with the other clones—indicating that this clone probably had been formed by fusion of two 3T3 cells and one HTC cell, or of one 2S 3T3 cell and one HTC cell. Both 3T3 "survivors" had a mode of 69 chromosomes, and narrow ranges. One of them had developed a single metacentric chromosome. Thus, since no marked chromosomal rearrangements occurred in the 3T3 parent as a result of the hybridization procedure, we conclude that almost all of the metacentric chromosomes in the hybrids are derived from the HTC parent.

AHH activity

Table 1 shows that the hydroxylase activity in 15 of the 16 randomly chosen hybrid clones was more inducible by BzAnth than was the enzyme in the 3T3 parent. The levels of maximally induced hydroxylase activities in hybrids 28, 11, and 29 were at least 2-fold, in hybrids, 24, 6, and 23 were more than 10-fold, and in hybrid 22 were more than 20-fold,

TABLE 2. *Failure of anti-aminotransferase antibodies to inactivate the aminotransferase-like enzyme activity found in 3T3 parent, 3T3-HTC hybrids, or 3T3 "survivor"*

Cell line	Absorbance at 331 nm	Protein (mg/ml)	Specific amino-transferase activity
HTC	1.70	5.8	4.2
HTC + Ab*	0.58	5.9	1.4
3T3	0.09	1.6	0.8
3T3 + Ab	0.10	1.7	0.8
Hybrid 7	0.80	8.4	1.4
Hybrid 7 + Ab	0.82	8.6	1.4
Hybrid 9	0.52	6.6	1.1
Hybrid 9 + Ab	0.60	6.6	1.3
Hybrid 12	0.59	5.8	1.4
Hybrid 12 + Ab	0.58	6.6	1.2
Hybrid 20	0.50	5.4	1.3
Hybrid 20 + Ab	0.51	5.8	1.2
3T3 "survivor" 1	0.05	1.4	0.5
3T3 "survivor" 1 + Ab	0.05	1.6	0.5

* Freshly-grown noninduced cells of each line shown were washed twice with phosphate-buffered saline, scraped loose from the tissue-culture dish, suspended in 0.1 M potassium phosphate buffer (pH 7.6) supplemented with 0.1 mM pyridoxal phosphate, and divided equally into two parts. To one part was added 1 μl of sheep anti-serum aminotransferase globulin (from rat) fraction. The extracts were incubated at 37° for 60 min, and assayed for remaining constitutive aminotransferase activity. *Ab*, antibody.

greater than that in the 3T3 parent cells. Two 3T3 marker chromosomes were present in clone 22. Thus, since clone 22 had double the 3T3 chromosomal complement and at least twice as much BzAnth-inducible hydroxylase activity as that in any other hybrid, the induction process in this clone appears to be 3T3 gene dose-dependent. The maximally inducible hydroxylase activities in hybrid 30 and in the 3T3 "survivors" were not significantly different from the activities (ranging from 1.5 to 3.0) in the BzAnth-treated 3T3 parent cultures. In none of the hybrids was hydroxylase induction by BzAnth significantly less than that found in the 3T3 parent. Except for clone 22, we see no obvious correlation between the magnitude of hydroxylase induction in the hybrids and the chromosomal contribution from either parent.

With cells in culture, we found (13) specific hydroxylase activities greater than 10 only in cells derived from liver, where the enzyme system induced by aromatic hydrocarbons or phenobarbital may reach specific activities of more than 100 (18, 19). HTC cells are of liver origin (5). Therefore, it is possible that in these hybrids, genetic information expressing hydroxylase induction in 3T3 cells is able to "activate" a pre-existing regulatory mechanism in the HTC parent. One possibility for testing this hypothesis is to look for hydroxylase induction by phenobarbital, a process occurring principally in hepatic cells or tissue (18–21). However, we found no hydroxylase induction in several clones by 5 mM of phenobarbital (not shown); this finding does not necessarily rule out this hypothesis, since responsiveness of enzyme induction in cultured cells to phenobarbital most likely is a complex phenomenon.

HTC homogenates mixed in various proportions with extracts from either 3T3 or clone-23 cells, and HTC cells grown with 3T3 cells in culture, produced only additive effects of hydroxylase activity either *in vitro* or in cell culture (not shown). No differences in the relative thermolability of the constitutive or BzAnth-induced hydroxylase activities from the 3T3 parent and from clone 23 were found when cellular homogenates were exposed to various temperatures without substrate or cofactors; more than 90% of each hydroxylase activity remained after 5 min at 45°, and less than 10% of each enzyme activity remained after 5 min at 55° (not shown).

Rate of hydroxylase induction and decay

The maximally induced hydroxylase activity was generally reached between 4 and 6 days in all of the hybrids; this rate of accumulated hydroxylase activity was strikingly similar to that found in the 3T3 parent. The rate of hydroxylase induction in these established cell lines is distinctly different from that found *in vivo* (21), or in hepatic (18), or nonhepatic (22) primary- or secondary-cell cultures, wherein hydroxylase induction by polycyclic hydrocarbons is always maximal in 24–48 hr. Hydroxylase induction in 3T3 cells or in any of the hybrids by BzAnth was completely blocked by 0.40 μM actinomycin D or 3.5 μM cycloheximide added initially; such a response is very similar to that seen with fetal-hamster secondary cultures (4, 22, 23) or with fetal-rat primary hepatocyte cultures (19). Actinomycin D, added to the 3T3 parent or hybrid clones 12 or 23 in which hydroxylase activity had been induced, caused a delay in the usual rate of decay of induced hydroxylase activity. This effect by actinomycin D was prevented by cycloheximide. The half-life of BzAnth-induced hydroxylase activity in the 3T3 parent or in several hybrids was 3–4.5 hr, whether the cells were grown in control medium alone, in the presence of cycloheximide, or in the presence of actinomycin D plus cycloheximide. All of these responses of hydroxylase activity to actinomycin D and cycloheximide and the rate of degradation of the BzAnth-induced enzyme are very similar to those described (4, 22, 23) for hamster-fibroblast cultures.

TAT activity

In marked contrast to an increased extent of hydroxylase induction by BzAnth in 15 of 16 hybrids, aminotransferase induction by dexamethasone was totally suppressed in all hybrids. Table 1 shows that all of the specific activities of aminotransferase ranged from 0.3 to 2.6 after dexamethasone treatment of the hybrids, and were not significantly different from their control aminotransferase activities or those in the 3T3 parent. The basal specific activity of aminotransferase in HTC cells in Table 1 was 18. Table 2 demonstrates that the quantity of monospecific antiserum, prepared against authentic rat aminotransferase (5, 27) and sufficient to inactivate about 67% of the control aminotransferase activity in HTC cells, had no effect on the aminotransferase activity from 3T3 cells, from one 3T3 "survivor," or from the four hybrids examined. It appears that the aminotransferase activity found in the hybrids is principally of 3T3 origin, and that the HTC enzyme is not expressed. This finding differs from the work of others, in which the noninducible aminotransferase in hybrids formed between rat Reuber hepatoma cells and 3T3 (24) or rat-liver epithelial cells (25) appears to be derived from both parents. It is unlikely that the chromosome bearing the aminotransferase gene(s) was lost in all of our hybrids, especially since about a 1S HTC chromosomal complement was usually present.

One possible explanation of our data is that an inhibitor of aminotransferase activity from HTC cells exists in the 3T3 parent. Various proportions of each of four mass "hybrid" cultures, the 3T3 parent, or clones 4 and 10 were mixed with extracts of HTC cells *in vitro*, and various amounts of 3T3 and HTC cells were grown together in culture; in each case, purely additive aminotransferase activity was found (not shown).

Another mechanism that could explain the absence of HTC-type aminotransferase in the hybrids is that the clones possess some factor that interferes not with the transcription but with the processing and/or translation of the aminotransferase message. If this function were transcription-dependent and turned over rapidly relative to aminotransferase mRNA, then blockage of transcription might result in a burst of aminotransferase activity. Aminotransferase activity is increased by blockage of RNA synthesis in both normal liver (26) and HTC cells (27). Accordingly, both mass "hybrid" cultures and clones 3, 5, 7, 12, 17, and 20 were treated with 3.6 μM actinomycin D for 3 hr, extracted, and assayed for aminotransferase activity. No enhancement of aminotransferase activity was observed in any experiment.

Finally, the possibility that the low aminotransferase activity in the hybrids might be caused by extremely rapid degradation of the enzyme was considered. Clones 23 and 9 were treated with dexamethasone, and cells were sampled hourly for aminotransferase activity for 7 hr. At no time was any increase in activity over baseline seen (not shown). We also found no decay of aminotransferase activity when

hybrid and HTC cell homogenates were mixed and held at 37° for 1 hr. Therefore, if the absence of HTC-like aminotransferase in the hybrids reflects rapid degradation of the enzyme, this degradative activity is totally lost in extracts from the hybrid cells. Further, the relative magnitude of induction should increase and the rate of synthesis become more rapid if only the decay rate of an induced enzyme is increased (28). We saw neither event in the experiments mentioned above.

DISCUSSION

Our data suggest that the induction processes for these two enzymes are expressed differently. Whether these differences represent dissimilar regulatory mechanisms on a molecular level is not known. We have confirmed the findings that aminotransferase induction by dexamethasone is suppressed in hybrids between hepatoma cells having inducible aminotransferase and other cell lines having no inducible aminotransferase (11, 24, 25). The apparent return of some aminotransferase induction in one such hybrid after loss of more than one-third of the chromosomes initially present (25) is further evidence for suppression of inducible aminotransferase activity by genetic material from the parent having the noninducible aminotransferase. On the other hand, if one assumes random chromosomal loss, hydroxylase induction cannot be repressed by one or more HTC chromosomes, since most of the hybrids contain about a 1S chromosomal complement.

In HTC–3T3 hybrids, what might increase hydroxylase induction 2- to more than 10-fold, compared with that in the 3T3 parent? (*a*) HTC genetic material is "activated," modified, or amplified in some manner by 3T3 gene products, resulting in HTC-specific induction-specific RNA, protein, and hydroxylase activity; (*b*) conversely, 3T3 gene(s) are changed in some way by HTC gene products, leading to an increase in 3T3-specific mRNA, protein, and hydroxylase activity; (*c*) heterogeneity within the 3T3 parent line exists such that the hybrids arose from individual 3T3 cells having a magnitude of hydroxylase induction the same as, to more than 10-fold greater than, that found as an average in the general 3T3 population; or (*d*) pre-existing post-transcriptional HTC or 3T3 material is used advantageously or more efficiently by induction-specific RNA or protein originating from either 3T3 or HTC gene(s). For example, perhaps the HTC-microsomal membranes can accommodate far more 3T3 "substrate-specific protein" (4) than can the 3T3 microsomes. Our study does not distinguish between these four general hypotheses. Among hybrids between 3T3 and human or hamster established lines not derived from liver or hepatoma, we have found that hydroxylase induction by BzAnth is never greater than that seen in the 3T3 parent (29). In this report, cell fusion is between one cell line having inducible hydroxylase and the other line having no detectable basal or inducible hydroxylase activity. Other combinations, such as a fusion between one parent having highly inducible hydroxylase activity and another parent having hydroxylase inducible to a lesser degree, would be of interest.

Hydroxylase induction by aromatic hydrocarbons in mice (21), with an associated change in the enzyme active site (14, 30, 31), is expressed as a simple autosomal-dominant trait, for which the *ah* locus has been proposed. It is interesting that hydroxylase induction by polycyclic hydrocarbons, a demonstrable autosomal-dominant trait *in vivo* (21), appears to be expressed dominantly, rather than suppressed, in fused-cell hybrids in culture.

We thank Betty Paul and Clarence Kelly (Birth Defects Institute of New York State, New York State Health Department, Albany, N.Y.) for their valuable technical assistance and Dr. Edward L. Kuff (National Cancer Institute, Bethesda, Md.) for his critical review of the manuscript. This work was presented at the Meeting of the American Society for Experimental Pathology, Atlantic City, N.J., April 1972.

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