Genetics of Somatic Mammalian Cells: Demonstration of a Human Esterase Activator Gene Linked to the AdeB Gene*

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Contributed by Theodore T. Puck, August 25, 1972

ABSTRACT Prototrophic hybrids formed from an adenine-requiring Chinese hamster cell and human fibroblasts uniformly display new esterase activity that differs from that of either parental cell in electrophoretic mobility and substrate specificity. The hybrids that grew in the selective medium and possessed the new esterase activity had a single extra chromosome that resembled a B-group human chromosome. When clones of such hybrid cells were cultured in nonselective medium, they rapidly reverted to inability to synthesize adenine, disappearance of the new esterase activity, and simultaneous loss of the extra human chromosome. Esterase activity like that of the hybrid is present in cells of various Chinese hamster, but not human, tissues. It is postulated that partial Chinese hamster esterase genes became inactive after long-term cultivation, and that, in the hybrid cell, a human activator gene linked to the adeB gene and located on a human B-group chromosome reactivated expression of these Chinese hamster esterase genes.

In previous papers of this series, linkage determination of human genes was described by fusion of human and auxotrophic Chinese hamster cells and growth in selective media in which only hybrids formed by fusion of both cell types develop into colonies. Loss of unnecessary human chromosomes permits linkage of other genes to the selective marker to be readily determined. The Chinese hamster ovary cell (CHO-K1) is particularly advantageous for this purpose because of the large number of genetic markers that are available (1) and because its hybrids with human cells lose unnecessary human chromosomes with unusually great rapidity (2). Two cases of human gene linkage revealed by this system have been described: a lethal antigen A₄ and lactate dehydrogenase A, and serine hydroxymethylase and lactate dehydrogenase B (3, 4). In this paper we establish linkage between a human adenine marker and a human gene that appears capable of activating specific Chinese hamster esterase activity when introduced into Chinese hamster cells.

METHODS AND MATERIALS
The cells used were CHO-K1 Chinese hamster cells, its auxotrophic mutants, and various human fibroblasts. The origin,

* No. XVI in a series "Genetics of Somatic Mammalian Cells." The preceding paper of this series is ref. 4.

properties, and mode of cultivation in vitro of these various cells have been described (4–7). The adeB mutant has a growth requirement for adenine or one of a variety of its precursors and is blocked in one of the steps between 5-phosphoribosyl pyrophosphate and 5-aminomimidazole-4-carboxylic acid ribonucleotide (1). Two kinds of growth media were used: complete medium consisting of F12 (8) supplemented with 10% fetal-calf serum, which supports growth of all cells, and selective medium, consisting of F12D supplemented with the macromolecular fraction of fetal-calf serum as described (2), in which only prototrophic cells can grow.

Ultraviolet-irradiated Sendai virus was used to promote hybridization (9). Single-cell plating was done by standard methods (5). The principles underlying selection of primary and secondary clones are the same as those discussed earlier (4).

The method used for isozyme analysis was similar to those described (4) and used the Canalco model 1200 Bath and the model 100 Constant Rate Power Source. After electrophoresis, the gels were removed from the column and soaked in 50 mM Tris buffer (pH 7.4) for 30 min before they were stained. When various inhibitors were used, they were added to this buffer and also to the staining solution. For each gel the staining solution for esterase consisted of 2.0 ml of 50 mM Tris buffer (pH 7.4), 1 mg of Fast Blue RR, and 0.1 ml of 1% substrate in 50% acetone. The diazo salt and buffers were equilibrated at 37° for 30 min before use, and the substrate was added to the solution just before insertion of the gel. α-Naphthylbutyrate and indoxylacetate usually produce best results when they are freshly prepared. Gels were incubated in the staining solution at room temperature for 45 min for α-naphthylacetate, and for 3–4 hr for α-naphthylbutyrate and indoxylacetate. The gels were then rinsed with distilled water and either photographed immediately or stored in 7.5% acetic acid.

The following CHO-K1 auxotrophic mutants with nutritional requirement as indicated were used: glyA (glycine); glyB (glycine or folic acid); glyC (glycine); glyD (glycine); adeB (adenine or hypoxanthine); ino⁻ (inositol); pro⁻ (proline); GAT⁻ (glycine + adenine + thymidine) (1). A hybrid clone formed by fusion of a human fibroblast and adeB mutant and grown continuously in the deficient medium was used most frequently in these experiments, and was named HAB-1. Subclones obtained from this clone after growth in complete medium for periods long enough to revert to auxotrophy were named HAB-1-R. The isozymes studied were: lactate dehydrogenase A (LDH-A, EC 1.1.1.27); lactate dehydrogenase B
RESULTS

Preparation of hybrid cultures

Human fibroblastic cells were hybridized with each of the following eight auxotrophic mutants of the CHO-K1 cell: glyA, glyB, glyC, glyD, adeB, ino−, pro−, and GAT−. On completion of fusion, the cells were cultivated in selective medium F12D (6), which does not support growth of any of the parental cells used in these hybridizations. The uncloned hybrid cultures cultivated in selective medium revealed modal chromosome numbers of 20–21 two weeks after fusion, in agreement with results previously reported (2).

Properties of hybrid cultures

Evidence for Linkage from Isozyme Assays. The hybridized cultures were harvested during the fourth week of growth after the initial fusion. Cell extracts were prepared, and isozyme patterns were determined for the following 10 enzymes:

- LDH-A
- LDH-B
- MDH-NAD
- MDH-NADP
- G6PD
- PGM
- IPO
- GOT
- GPI
- Esterase

(LDH-B, EC 1.1.1.27); NAD-dependent malate dehydrogenase (MDH-NAD, EC 1.1.1.37); NADP-dependent malate dehydrogenase (MDH-NADP, EC 1.1.1.40); glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49); phosphoglucomutase (PGM, EC 2.7.5.1); glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1); indophenol oxidase (IPO); glucose phosphate isomerase (GPI, EC 5.3.1.9); and esterase.

The following chemicals were used: α-naphthylacetate, α-naphthylbutyrate, indoxylacetate, eserine, p-chloromercuribenzoate (Sigma); and 1,5-bis-(4-allyl-dimethyl ammonium phenyl)pentane-1,3-dibromide (BW) (BW284C51, Burroughs-Wellcome Co.).

Table 1. Evidence for linkage between the adeB marker and the three extra esterase bands

<table>
<thead>
<tr>
<th>Cell phenotype</th>
<th>adeB</th>
<th>+</th>
<th>+</th>
<th>−</th>
<th>−</th>
<th>+</th>
<th>−</th>
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<tbody>
<tr>
<td>No. of primary and secondary clones found</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>30</td>
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against three different substrates: \( \alpha \)-naphthylacetate, \( \alpha \)-naphthylbutyrate, and indoxylacetate. The zymograms obtained from the use of the three different substrates with the three cell types are shown in Fig. 2, in which the unique bands present in the hybrids have been designated I, II, and III. It is apparent that bands I, II, and III are electrophoretically different from those of either parental cell. In addition, the substrate specificity of these three bands is different in that they appear active with either \( \alpha \)-naphthylacetate or \( \alpha \)-naphthylbutyrate, whereas the human and Chinese hamster enzymes are active only toward \( \alpha \)-naphthylacetate. None of the three unique isoforms is active toward indoxylacetate.

The three esterase bands unique to the hybrid also exhibited a different pattern of sensitivity to inhibitors from those of each of the parental cells (Fig. 3). All of the bands from each parental cell were unaffected by 0.1 mM eserine, a specific inhibitor of choline esterases (10), and all except the most strongly cationic of the bands of the Chinese hamster cell were unaffected by 0.1 mM p-chloromercuribenzoate, a specific inhibitor of arylesterases (11). The activity of band III of the hybrid cells, however, disappeared on addition of eserine, while band II was eliminated completely and band I was partially weakened by addition of p-chloromercuribenzoate. An identical pattern of inhibition of these three bands was obtained by the use of either \( \alpha \)-naphthylacetate or \( \alpha \)-naphthylbutyrate as substrate.

These results indicate that the three unique bands represent enzyme activities not found in either parental cell alone; that more than one new enzymatic activity and, therefore, perhaps more than one new kind of protein may be present in the hybrid; that band II could be any of the arylesterases, which preferentially hydrolyze aromatic esters; and that band III, which is sensitive to the specific inhibitor, eserine, might be either cholinesterase (EC 3.1.1.8) or acetylcholinesterase (EC 3.1.1.7). The effect of 5 \( \mu \)M BW, which inhibits acetylcholinesterase (12), was also tested, and band III was insensitive to it. Hence, band III might represent a cholinesterase activity.

**Origin of the New Esterase Activities of the Hybrid.** Our experiments raise the question about the origin of the three new esterase bands present in the hybrid. Since these bands occur in neither parental cell, regulatory genes in one of the parents might have resulted in phenotypic expression of genes in the other that were previously suppressed. If these esterase bands unique to the hybrid cell represent proteins formed by inactive structural genes present in only one of the parental cells, it should be possible to identify similar bands in cells taken from one of the parental species but not in the other. Since the parental CHO-K1 culture used in these hybridizations has been cultivated for more than a decade and has lost some of its original chromatin content (13), we studied the esterase activity from a more recent Chinese hamster ovary culture, designated CHO-II. This culture was initiated from a biopsy taken 6 months previously and contains an apparently normal karyotype. The zymogram obtained from this culture is shown in Fig. 4 (gel channels 1–6), and demonstrates the presence of three bands, marked I, II, and III, which appear to correspond electrophoretically to those present in the human-adeB hybrids (although band III in the CHO-II cell has a somewhat lower intensity than that in the hybrid). These three bands in the CHO-II cells also possess substrate specificity and inhibitor sensitivity identical to those of the three unique bands found in the hybrid cell.

Direct tests were also made of esterase activity in cells from a Chinese hamster biopsy. Ovaries were removed from

![Fig. 3. Demonstration of inhibitor specificity of the new esterase bands. Esterase zymograms in channels 1–3 are from human; 4–6 are adeB; and 7–13 are the hybrid clone HAB-1. The substrate used in channels 1–9 is \( \alpha \)-naphthylacetate, and that in 10–13 is \( \alpha \)-naphthylbutyrate. PCMB, p-chloromercuribenzoate; BW, 1,5-bis-(4-allyl-dimethyl) ammonium phenylpentane-1,3-dibromide (5 \( \mu \)M).](image-url)

![Fig. 4. Demonstration of the presence of the new esterase bands in a short-term culture made from a biopsy of a Chinese hamster ovary, CHO-II (channels 1–6), and in a direct biopsy without cultivation from Chinese hamster ovary (channels 7–12). Channels 1–3 and 7–9 use \( \alpha \)-naphthylacetate as substrate while 4–6 and 10–12 use \( \alpha \)-naphthylbutyrate. Channels 1, 4, 7, and 10 have no inhibitors; eserine was present in channels 2, 5, 8, and 11; and p-chloromercuribenzoate was present in channels 3, 6, 9, and 12.](image-url)
young females, homogenized and assayed for esterase. The results are shown in Fig. 4 (gel channels 7–12). Although the patterns are more complex than those shown in the tissue culture cells, bands corresponding to those designated I and II could be identified by their electrophoretic mobility, substrate specificity, and inhibitor sensitivity. A band corresponding to band III in the hybrid cell was seen in some preparations in fairly low activity, though not in others. When present it was also sensitive to eserine, like band III in the hybrid cell. Similarly, esterase bands resembling those of bands I and II and occasionally III in the hybrid cell were readily demonstrated in cells from other tissues like lung, spleen, muscle, heart, and testis and from a long-term culture of Chinese hamster lung cells.

Conversely, several different kinds of human cells were examined for esterase activity, and no bands were found corresponding to the activities labeled I, II, or III in the hybrid cell. The different types of human preparations tested included erythrocytes, lymphocytes, amniotic fluid cells taken directly or cultivated for several weeks in tissue culture, skin fibroblastic cells in early passages, a virus-transformed skin cell culture, and HeLa cells. These results appear to justify, at least provisionally, the conclusion that bands I, II, and III are of Chinese hamster cell origin, are inactive in CHO-K1 cells, and are activated in the human–adeB hybrid by virtue of an activator gene present on the human chromosome that also supplies the adeB activity.

**Reversibility of the Activating Action.** If a Chinese hamster structural gene has been activated by a gene carried on the human chromosome that also supplied adeB activity, the question arises whether this gene activation is reversible. Evidence for such reversibility is implied in the experiments described above in which all 30 hybrid clones examined had simultaneously lost both the new esterase bands and the adeB activity after 1 month’s growth in complete F12 medium, and at the same time lost an extra chromosome from their karyotype. If loss of the extra chromosome containing the esterase activating gene results in loss of esterase expression, activation of Chinese hamster esterase would be a reversible action that requires the continued presence of the activating gene. In a further experiment, one of the revertant clones that had lost the extra esterase bands and the adeB marker as a result of growth in the complete, nonselective F12 medium was again hybridized with a human cell and grown for 3 weeks in deficient F12D medium. An esterase assay demonstrated that the three extra bands had again appeared.

**Preliminary Karyotypic Analyses.** Karyotypes of primary clones isolated after fusion between human and adeB cells have so far been analyzed only by measurement of chromosomal arm lengths. A typical karyotype representative of the great majority of these clones is presented in Fig. 5. It contains 21 chromosomes, one more than that of the Chinese hamster adeB parent. By visual inspection, 20 chromosomes of the hybrid can be identified with the corresponding chromosome of the original adeB karyotype. The extra chromosome placed in the column marked *Hu* resembles one of the human B-group of chromosomes. This identification based on total chromosome length and centromeric position alone is tentative. More definitive studies with the chromosome banding technique are now in progress.

The extra hybrid chromosome that resembles the human B-group was present in most of the cells examined. Some hybrid cells were found that possessed only 20 chromosomes but contained the adeB marker. These cells appear to have lost one of the Chinese hamster chromosomes, but they have retained the extra B-group-like human chromosome. A small proportion of the hybrid cells have karyotypes slightly different from the typical pattern presented in Fig. 5, occasionally ex-
hibiting one or two additional chromosomes or failing to exhibit a clearly identifiable human, B-group-like chromosome. We provisionally interpret such cells as resulting from chromosomal rearrangement.

Karyotypic analysis of the 20 clones isolated from the HAB-1 clone that had been transferred to complete F12 medium supported the above tentative conclusion. These clones, which had lost both the extra esterase bands and the adeB marker, were found uniformly also to have lost the human B-group-like chromosome that was originally present in the HAB-1 clone. The karyotype of a typical clone of this kind, named HAB-1-R1, which appears to be identical to that of the original adeB parent, is shown in Fig. 5.

**DISCUSSION**

Our data suggest a regulatory genetic operation that activates specific esterase functions. Since this regulatory action originates in a human chromosome and operates on a Chinese hamster chromosome that contains the Chinese hamster structural gene for esterase activity, a transferable gene product would appear to carry the regulatory activity. The regulatory activity is also linked to the gene controlling adeB activity. The regulatory action of the esterase activities is reversible, so it would appear to resemble induction phenomena described in bacterial systems (14). The exact sequence of biochemical steps involved between acquisition of the regulatory gene and appearance of esterase activity in the cells requires detailed study.

The linkage groups described here are different from those containing genes for pro, LDH-B and glyA, glyB, glyC, glyD, ino, GAT, LDH-A and A\textsubscript{L}, MDH-NAD, MDH-NADP, G6PD, PGM, IPO, GOT, and GPI. More than one structural gene may be involved in this regulatory unit, and other human chromosomes may contain a regulatory gene like that demonstrated here. While the possibility must be considered that one or more of the I, II, and III bands may arise from cross-polymerization of Chinese hamster and human protein, it appears more likely that all of these bands represent Chinese hamster proteins exclusively since similar bands can be elicited from Chinese hamster cells alone. Moreover, the hybrid cell has never exhibited an esterase band characteristic of a pure human esterase.

The evidence also suggests that the new linkage groups are carried on a single human chromosome, which may be one of the human B-group chromosomes. Secure identification of the human chromosome is of interest in view of the existence of two different human diseases each due to a deletion in one of the B-group chromosomes. The 4p-syndrome is due to a partial deletion of the short arm of chromosome 4 (15), while the cri-du-chat syndrome is characterized by a deletion in chromosome 5 (16). Cells from such patients could be compared with normal human cells with respect to the following properties: the amount of adenine biosynthetic activity in each cell; the amount of esterase activity exhibited toward \(\alpha\)-naphthylacetate and \(\alpha\)-naphthylbutyrate; the frequency of occurrence of ade\textsuperscript{+}B hybrids after fusion with ade\textsuperscript{-}B Chinese hamster cells; the frequency with which such hybrids that are selected with respect to the ade\textsuperscript{+}B activity also contain the extra esterase bands described here; the frequency with which ade\textsuperscript{+} hybrids with and without the new esterases contain the normal or the partially deleted human chromosome. Such studies, currently in progress, may yield important contributions to the genetic analysis of the human B-group chromosomes.

Discovery and characterization of regulatory genes is of primary importance in unraveling mechanisms of mammalian differentiation. A genetic regulator element in human somatic cells has been described by Klebe et al. (17) that extinguishes the activity of a kidney-associated esterase, in contrast to our system in which activation is obtained. Peterson and Weiss have described a system for induction of synthesis of mouse albumin by hybridizing mouse fibroblasts, which normally do not produce this protein, with rat hepatoma cells (18). The selective techniques that are made possible by the Chinese hamster auxotrophic mutants should facilitate studies of this kind.

We appreciate the competent technical assistance of Miss Judy Hartz. This investigation is a contribution from the Eleanor Roosevelt Institute for Cancer Research and the Department of Biophysics and Genetics (No. 507), University of Colorado Medical Center, Denver, Colo. The investigation was aided by an American Cancer Society Grant No. VC-81B. T. T. P. is an American Cancer Society Research Professor.