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Presence of Two Forms of Fumarase (Fumarate Hydratase E.C. 4.2.1.2) in Mammalian Cells: Immunological Characterization and Genetic Analysis in Somatic Cell Hybrids. Confirmation of the Assignment of a Gene Necessary for the Enzyme Expression to Human Chromosome 1

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Two major forms of fumarate hydratase have been resolved in extracts prepared from a wide variety of mammalian cells by electrophoresis. Fractionation experiments with human and mouse cells suggest that one form (the slower migrating) is localized in the mitochondria, whereas the other form is predominant in the cytoplasm. Analysis of the segregation of the enzyme forms in human–mouse somatic cell hybrids indicates that a gene(s) necessary for the expression of both forms can be assigned to human chromosome 1 (confirmation of a previous assignment by van Someren et al., *1974). Electrophoretic analysis suggests that the two forms may be interrelated. Furthermore, they both exhibit identical reactivity toward anti-fumarate hydratase antiserum. It is suggested that a modification of one form may occur* in vivo *and that the modification may be important in determining the intracellular localization of the enzyme.*

KEY WORDS: fumarate hydratase; mammalian cells; somatic cell hybrids; enzyme expression.

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INTRODUCTION

Fumarase (fumarate hydratase, E.C. 4.2.1.2) is an enzyme of the citric acid pathway. It has been assumed generally that it is predominantly localized in the mitochondria (see Munn, 1974). However, its presence has been detected in mature anucleate erythrocytes (see Schweiger, 1962), which lack most mitochondrial enzymes. Furthermore, Lin *et al.* (1971) reported that a considerable fraction of the total enzyme activity is found in high-speed (post-mitochondrial) supernatants of tissue homogenates. Their report further indicated that the enzyme when purified from animal tissues may contain several different molecular species each having enzymatic activity. Previous investigations on enzymes purified from pig heart suggested a tetrameric structure comprising four identical polypeptide subunits of mol wt 48,500 (Kanarek *et al.*, 1964). However, isoelectric focusing of dissociated subunits (Penner and Cohen, 1971) demonstrated that several different types existed, each capable of reassociating to form an active enzyme. This behavior suggested the existence of several different genes which code for subunit polypeptides of different primary structure or the occurrence of extensive posttranslational modification of a single gene product *in vivo*.

We have employed electrophoretic and immunological procedures to examine the forms of fumarate hydratase present in human and mouse cultured cells. This represents an extension of previous studies concerned with establishing procedures for the genetic analysis of (nuclear-coded) mitochondrial components in human–mouse somatic cell hybrids (van Heyningen *et al.*, 1973, 1974; Craig, 1975). Our observations indicate that two major forms of fumarate hydratase exist in cultured animal cells, one localized predominantly in mitochondria and the other in the cytosol. Analysis of the segregation of both the mitochondrial and cytoplasmic human forms of fumarate hydratase in human–mouse hybrid lines and subclones suggests that the genetic information necessary for the expression of both enzyme forms resides on human chromosome 1. This is consistent with the existence of two (or more) syntenic structural genes, one necessary for the expression of the cytoplasmic form, the other for the mitochondrial form. Alternatively, a single structural gene could exist whose product is subsequently modified. The mitochondrial and cytoplasmic forms cannot be distinguished on the basis of their reaction with antisera raised against a commercial preparation of fumarate hydratase. Furthermore, when cell extracts from a variety of different species were compared, interspecific differences in electrophoretic patterns (when observed) were never confined to one form; the migration distances of both were affected to a qualitatively similar extent. We therefore favor the latter interpretation, namely that of modification.

MATERIALS AND METHODS

Cell Lines and Hybrids

The mouse cell lines and hybrids employed have been described in detail previously (van Heyningen *et al.*, 1973; Povey *et al.*, 1974). Other cell lines used were the following:

XTC-2, a cell line derived from *Xenopus laevis*.
SC-9, a cell line derived from ovary tissue of marsupial mouse (*Sminthopsis*).
DON, a cell line derived from Chinese hamster.
WFU-5, a rat hepatoma line (Deschatrette and Weiss, 1974).
D98, an 8-azaguanine-resistant human cell line (Syzbalski *et al.*, 1962).
RI, a primary fibroblast line from rabbit skin.
CI, a primary fibroblast line from chimpanzee.

Cells were grown as monolayers on glass in RPMI 1640 (Biocult Laboratories) supplemented with glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) plus 10% fetal calf serum.

Preparation of Extracts

Cell pellets obtained after trypsinization were washed once in phosphate-buffered saline and stored in liquid nitrogen. They were suspended before use in sodium phosphate buffer, *p*H 7.0 (50 mM), containing ethylenediaminetetraacetic acid (1 mM), 2-mercaptoethanol (1 mM), and NADP (20 μM) to 10^8 cells/ml. The suspension was sonicated (MSE ultrasonicator) at maximal output three times for 15 sec each and subsequently clarified by centrifugation for 1 min in a Beckman microfuge.

Electrophoretic Procedure

The procedure employed for cellulose acetate (Cellogel) electrophoresis has been described previously (Craig, 1973). The buffer employed for presoaking the gel and for electrophoresis was 2.4 mM trisodium citrate–17.2 mM sodium dihydrogen phosphate adjusted to *p*H 7.0 with NaOH before dilution to final volume. The optimum *p*H range for separation of the two forms observed in cell extracts was *p*H 5.5–7.0. Extracts (0.5–1.5 μl) were applied 6–8 cm from the cathode and electrophoresis was continued for 1.5–2 hr. Generally, 7.8 by 15 cm gels were used sideways, the samples being applied about 4 cm from the cathode. The observed current under these conditions was about 8 mA at a voltage setting of 150. Fumarate hydratase activity was detected by

a procedure modified from that described by Brewer (1970). The staining mixture (0.5 ml/gel) was prepared from 0.2 ml sodium phosphate buffer, *p*H 7.0 (0.1 M), 0.2 ml fumaric acid (1 M) previously adjusted to *p*H 7.0 with NaOH, 46 μl NAD (50 mM), 50 μl thiazolyl blue (1 mg/ml), and 4 μl of malate dehydrogenase (approximately 5000 units/ml) obtained from Boehringer (London). The gels were stained for 10–15 min in the dark at 37 C. They were then removed and washed in running water to remove the nonreduced thiazolyl blue. The specificity of the staining reaction was checked by omitting fumarate from the reaction mixture. Under these conditions, faint bands could be detected after prolonged staining. The specific staining pattern of these suggested that they resulted from lactate dehydrogenase activity. However, they were not sufficiently intense to be detectable in most photographs and did not constitute a problem in the interpretation of fumarase banding patterns.

Spectrophotometric Assay

The activity of fumarate hydratase in extracts was assayed by the spectrophotometric procedure described by Hill and Bradshaw (1969) and related to the protein content determined by the Lowry procedure using bovine serum albumin as a standard.

Isoelectric Focusing

The isoelectric focusing procedure employed was based on that described by Penner and Cohen (1971). Samples of fumarate hydratase from the human cell line D98 were prepared by sonicating 2.5 ml of a cell suspension (10^8 cells/ml) at maximal output (MSE ultrasonicator) for four periods of 15 sec in sodium phosphate buffer, *p*H 7.0 (10 mM), EDTA (0.5 mM), 2-mercaptoethanol (0.5 mM), and NADP (0.4 μM). The disrupted cell suspension was adjusted to *p*H 5.2 with sodium acetate, *p*H 4.0 (1 M), and then centrifuged at 1500*g* for 20 min at 4 C. The supernatant was made 65% saturated with respect to ammonium sulfate by addition of the solid and gently stirred overnight.

The supernatant obtained after subsequent centrifugation at 12,000*g* for 30 min at 4 C contained no detectable fumarate hydratase activity and was discarded. The pellet obtained from 2.5×10^8 cells was resuspended in 2.5 ml of sodium phosphate buffer, *p*H 7.0 (5 mM), containing 1% (w/v) glycine and extensively dialyzed at 4 C against at least two changes of the same buffer. The extract, after clarification at 12,000*g* for 30 min, was used for electrofocusing. It was first mixed with ampholines and subsequently included in the stepwise sucrose gradient of the column. Focusing was effected for 3 days at

500 V in the presence of 1% (w/v) ampholines of the *p*H range 4–8. Temperature was stabilized by circulating of tapwater at 15 C.

Immunological Characterization

Antifumarase sera were prepared in mice and tested on Ouchterlony diffusion plates made with 1.5% (w/v) agar as previously described (van Heyningen *et al.*, 1973). At each primary or booster injection, 22–36 units of activity (60–100 μg of protein) was given to each animal. The antigen employed was commercially prepared fumarate hydratase from pig heart (Sigma, London, Ltd.). After extensive washing with two changes of 0.1 M sodium phosphate buffer, *p*H 7.0, over a period of 24 hr, the plates were stained for fumarate hydratase activity with the mixture as described above.

Immunoelectrophoresis was effected on cellulose acetate strip by coupling the electrophoretic procedure already described (0.5-μl sample) with a subsequent diffusion for 24 hr against 5 μl of antiserum. This was applied to the unstained gel immediately after the electrophoretic separation. It was distributed as a thin vertical line parallel to the direction of electrophoretic separation and between 0.5 and 1.0 cm from the expected position of the enzyme, the approximate location of the fumarate hydratase being first determined by staining a duplicate section of the gel. Washing and staining of the cellogel were carried out as described for the Ouchterlony plates.

Cell Fractionation

Two alternative procedures were employed for cell fractionation. A relatively crude fractionation was achieved by homogenizing 0.1 g wet weight of cells (3×10^7 cells) in 0.1 ml buffer containing mannitol (0.25 M). Cell breakage was achieved with a teflon on glass Potter homogenizer. The unbroken cells and nuclei were removed by centrifugation (750*g*) and the supernatant was saved. The pellet was resuspended to the original volume in buffer, rehomogenized, and centrifuged. The combined supernatants were then centrifuged at 9000*g* for 30 min to provide a pellet containing mitochondria and a supernatant containing cytosol enzymes. The latter was clarified by spinning at 30,000*g* for 1.5 hr. The mitochondrial pellet was washed in isolation buffer and then sonicated in a minimal volume of suspension buffer.

Alternatively, fractionation was performed as above except that 10^{-4} M phenyl methyl sulfonyl fluoride (PMSF) was included in the isolation buffer and the crude mitochondrial pellet was further purified by centrifugation on a 15–30% sucrose density gradient. The mitochondrial pellet was suspended in isolation buffer to about 25 mg wet weight/ml and layered on top of

preformed gradients of 20 ml. After centrifugation at 40,000*g* for 1.5 hr, 40-drop fractions were collected and assayed for cytochrome *c* reductase (Rabinowitz and de Bernard, 1957) as a marker of mitochondrial activity. Tubes corresponding to the peak of enzyme activity were pooled and the contents were diluted with an equal volume of isolation buffer. Mitochondria were pelleted at 10,000*g* for 30 min. The final mitochondrial pellets corresponding to 3×10^8 cells of starting material were suspended in 0.1 ml of cell buffer and sonicated as described for cell extracts.

RESULTS AND DISCUSSION

Differences in the electrophoretic patterns of fumarase extracted from human and Chinese hamster cultured cell lines have been reported and a provisional assignment of a gene responsible for the presence of human fumarase activity in hybrid cells has been made to human chromosome 1 on the basis of synteny with other, previously assigned, enzyme markers (van Someren *et al.*, 1974). However, no detailed analysis of the electrophoretic profile was made, and although more than one band of activity was found in extracts from both parental cells the possible existence of multiple forms of the enzyme was not considered.

Electrophoresis and staining of human and mouse extracts under the conditions described above showed clear differences between their respective migration patterns. Both were characterized by the presence of two predominant bands (see Figs. 1a and 1b). (Note: if the extracts are stored and reused several times the discrete banding becomes more diffuse.)

The presence of two major bands of activity suggested that more than one structural gene may be involved. Alternatively, the diversity could result from heterozygosity, or from posttranslational modification of a single gene product. Heterozygosity, however, is unlikely to generate only two separate and distinct bands of activity, as the available evidence suggests that the enzyme is comprised of 4 subunits. In general, subunits produced by different alleles would be expected to interact and therefore produce bands of intermediate mobility. Furthermore, the cell line (1R) which gives the characteristic two-banded pattern on electrophoresis is derived from an inbred mouse strain (C_3H).

By analogy with some other mitochondrial enzymes which possess cytoplasmic counterparts, we investigated the possibility that the two bands of fumarase activity represented two distinct forms of the enzyme which were localized intra- or extramitochondrially, respectively.

Mitochondrial and supernatant fractions prepared from both human and mouse cell lines (see Fig. 2) by differential centrifugation were found to retain predominantly either the slower or faster form of the enzyme detected

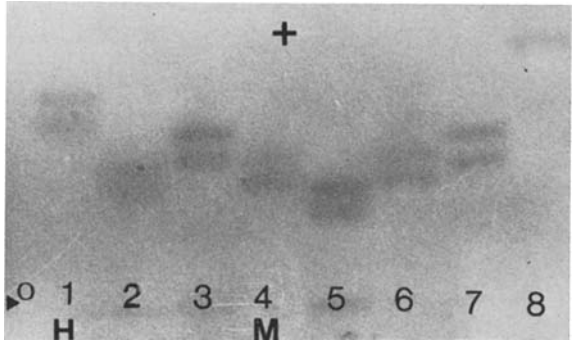

Fig. 1a. Photograph of the fumarate hydratase activity detected after electrophoresis and staining of cell extracts from a variety of species. Human (D98) and mouse (1R) samples are indicated by the letters H and M, respectively. The other cell extracts examined were (2) chimpanzee, (3) rabbit, (5) rat, (6) Chinese hamster, (7) marsupial, and (8) *Xenopus*. O represents the application point of the samples.

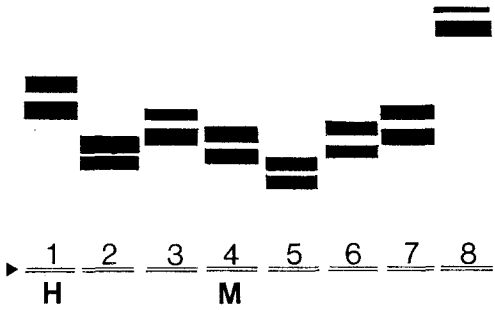

Fig. 1b. Diagrammatic representation of bands in Fig. 1a.

in cell extracts. Staining of the cellulose acetate strips after electrophoresis of cytoplasmic fractions generally resulted in a single discrete band of activity. Although the mitochondrial fraction demonstrated one major slowly migrating band, activity was also detected in some preparations in an equivalent position to that observed for cytoplasmic enzyme.

Mitochondrial fractions prepared by sucrose gradient fractionation were largely free of the extra band of activity, suggesting that the faster-migrating form was due to contaminating cytoplasmic material. Thus fumarate hydratase in the cultured cells examined is present in at least two major forms; the faster migrating (lower *p*K) predominates in the supernatant, whereas the slower (high *p*K) form is localized in mitochondrial preparations. This pattern is similar to that observed for several other mitochondrial enzymes that have

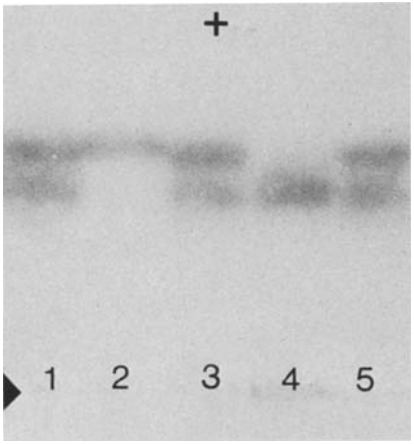

Fig. 2. Fumarate hydratase activity in mitochondrial and supernatant fractions of the human cell line D98. Samples 1, 3, and 5 were whole cell extracts, sample 2 was the cytoplasmic fraction, and sample 4 was the mitochondrial fraction. Mitochondrial and cytoplasmic fractions were prepared by differential centrifugation as described in Materials and Methods.

cytoplasmic counterparts. In some of these cases, there is evidence that the distinct forms are coded for by different genes (van Heyningen *et al.*, 1973; 1974; Craig, 1975).

It has been observed (Meera Khan, 1973; van Heyningen *et al.*, 1973; Craig, 1975) that although the enzymes localized in the cytoplasm of mouse, hamster, and human can frequently be distinguished under conditions of electrophoresis on cellulose acetate, *p*H 7.0–8.5, mitochondrial forms do not show much interspecific variation. We have therefore examined the electrophoretic profile of fumarate hydratase from a variety of cultured animal cell lines to assess the relative variations in the mitochondrial and cytosol forms. The most striking observation (see Figs. 1a and 1b) is that, although considerable interspecific variation in the mobility of the enzyme was observed, all the eutherian mammals and the one marsupial examined had a characteristic two-banded pattern, the activity being approximately equally distributed between the two bands. Samples from an amphibian (*Xenopus*) also exhibited two bands of activity. However, the slower form was strongly predominant. Furthermore, in spite of the considerable overall differences in migration rates between species, the relationships between fast- and slower-migrating bands (i.e., the distance separating them) remained remarkably constant. It should be borne in mind that some increase in separation is likely to occur with increasing total migration distance. A quantitative analysis of the relative migration distances has not been attempted because the conditions affecting migration of proteins vary across the cellogel strip during electrophoresis (see Kohn, 1968). Even when closely related mammals were examined, e.g., man and chimpanzee, or the various rodents, electrophoretic differences between species were never observed to be solely confined to one

band, although considerable overall variation was observed between the profiles for samples from closely related species.

Analysis of Fumarate Hydratase in Human–Mouse Somatic Cell Hybrids

Extracts from 11 independently derived human–mouse hybrids (representing four different parental cell type combinations) were examined by electrophoresis for the presence of human enzyme. Nine exhibited patterns of activity similar to those of the mouse control extract. In two hybrids (H22 and HORL), additional bands of activity were observed at positions between those normally observed in mouse and human marker extracts. These additional bands of activity are presumed to represent hybrid molecules of enzyme formed by interaction of human and mouse subunits. In hybrids expressing human activity, the distinct bands in the position(s) expected for the parental type human enzymes were absent (see Figs. 3a and 3b). This situation has been observed in the electrophoretic profiles of many enzymes which can form interspecific hybrid molecules and is presumed to result from a relatively low concentration of human enzyme subunits in the hybrids. This in turn probably reflects the effects of gene dosage and the low ratio of human : mouse

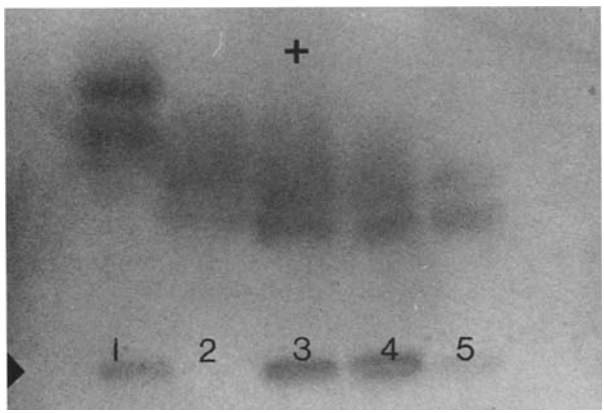

Fig. 3a. Photograph of the banding pattern obtained after electrophoresis of extracts and subcellular fractions from the human–mouse hybrid cell line H22 followed by staining for fumarate hydratase. Samples: (1) human (D98) cell extract, (2) hybrid supernatant fraction, (3) hybrid whole cell extracts, (4) hybrid mitochondrial fraction, and (5) mouse (1R) cell extract. The mitochondria were purified on sucrose density gradient, as described in Materials and Methods, before the enzyme was liberated by sonication.

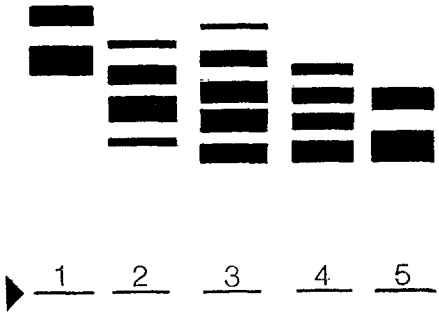

Fig. 3b. Diagrammatic representation of the bands observed in Fig. 3a. One possible interpretation of the observed distribution of bands which is consistent with the proposed tetrameric structure of the enzyme is that the hybrid mitochondrial fraction contains the following forms: M_4, M_3H, M_2H_2, MH_3, and H_4 where H and M represent the human and mouse subunits of the mitochondrial (slower-migrating) enzyme. The effects of gene dosage would predict that these would be present in decreasing concentration; e.g., if the ratio of mouse to human subunits was 2:1, the relative concentrations of MH_3 and H_4 would be one-eighth and one-sixteenth that of M_4 and probably would not be detected. Similarly, the supernatant prepared from the hybrid would contain m_4, m_3h, m_2h_2, mh_3, and h_4 where h and m represent the human and mouse subunits of the cytoplasmic (faster-migrating) enzyme. Similar considerations would obtain for the relative concentrations of the various forms. Note that the hybrid supernatant also contains a slower-migrating band than the expected m_4. This probably represents contamination with some mitochondrial material. For further discussion, see text.

copies of a particular chromosome. Even when one copy of a human chromosome is retained, at least two or three copies and as many as six copies of the equivalent mouse chromosome can be present in the same cell (see also Nabholz *et al.*, 1969; van Heyningen *et al.*, 1973).

Because of their close juxtaposition, complete resolution of the discrete bands of activity was not possible in some of the profiles observed for hybrids expressing both human and mouse fumarate hydratase. If fresh extracts are employed, the hybrid pattern appeared to be comprised of five or six bands (see Figs. 3a and 3b). Furthermore, fractionation (including sucrose gradient purification of mitochondria) of one hybrid presumed to contain human fumarase (H22) showed three slow-migrating bands of activity in mitochondrial preparations, whereas the supernatant was characterized by three or four faster, anodally migrating components. A possible interpretation of these patterns is presented in the caption of Fig. 3b.

The segregation of human fumarate hydratase has been examined in three extensively karyotyped and biochemically characterized subclones of HORL (one of the two hybrid lines found to express this enzyme) (see Table I). Human enzyme activity was detected in two subclones, both of which

Table I. Karyotypic Data on Hybrid Lines and Subclones[a]

Designation of hybrid or subclone	Human chromosomes	Human fumarate hydratase
HORL 4.1		
Subclone 1	1,3,11,13,15,22,X	+
2	1,3,11,13,15,22,X	+
9	11,15,X	−
2W1	7,13,21,X	−
HORP 24	4,7,11,12,14,15,21,22,X	−
3W4	7,10,11,12,14,15,X	−
H22	1,7,11,14,15,19,21	+

[a] The bulk of karyotypic data have been published previously (Povey *et al.*, 1974; van Heyningen *et al.*, 1975). Data on 3W4 are from Bobrow and Heritage (personal communication, 1975).

contained the following human chromosomes: 1, 3, 11, 13, 15, 22, X, and possibly 18. Human fumarate hydratase was absent from the third subclone, which had lost chromosomes 1, 3, 13, and 22 (see table). Furthermore, human fumarate hydratase was not detected in the hybrid lines 2W1 and HORP 24, which retained apparently intact chromosomes 13 and 22, respectively (see van Heyningen *et al.*, 1974). Thus only chromosomes 1 and 3 are present consistently in hybrids expressing human fumarate hydratase. The other hybrid line expressing human enzyme (H22) has been karyotyped and also found to retain chromosome 1. However, chromosome 3 was not observed in any of ten metaphases examined in detail (Povey *et al.*, 1974; Bobrow and Heritage, personal communication, 1975).

The enzyme analysis on the hybrids and subclones is also entirely consistent with the assignment of the gene(s) responsible for the expression of human fumarase to chromosome 1. Of 38 enzymes analyzed representing markers assigned to 14 chromosomes, only peptidase C and mutase 1 were found to be present in the same two hybrids and subclones (of 11 primary lines tested) as human fumarate hydratase. Both markers have previously been assigned to chromosome 1 (see New Haven Conference, 1973).

The enzyme profiles for the hybrid lines and subclones retaining human fumarate hydratase activity were similar and there was no evidence of bands other than those equivalent to the mouse parent in any other lines. This strongly suggests that, as human subunits were present in both mitochondrial and supernatant fractions from H22, chromosome 1 is necessary for production of both mitochondrial and supernatant forms of the enzyme.

Immunological Characterization

Antisera to pig heart fumarate hydratase were raised in mice as part of a more extended survey on several mitochondrial enzymes. Sera from four mice were examined and found to contain antibody which cross-reacted with human fumarate hydratase (present in extracts prepared from heart mitochondria). No spurs were observed at the intersection of precipitin lines formed between antiserum and adjacent wells containing human or pig antigens at equal enzyme activity. The formation of precipitin lines containing fumarate hydratase was detected by staining Ouchterlony plates for enzyme activity (replacement of fumarate in staining mixture by lactate did not elicit staining of the precipitin bands and therefore the patterns normally observed do not represent nonspecific staining). The antiserum chosen for subsequent studies had no detectable cross-reacting activity with fumarate hydratase present in mouse extracts.

The reaction of the antisera to the supernatant and to the mitochondrial forms of human fumarate hydratase was examined. Samples of mitochondrial or supernatant extracts from the human cell line D98 were adjusted to equal enzyme activity and diffused on Ouchterlony plates against the antiserum. About equal reaction against both antigens was observed (estimated from position of precipitin line) and no spurs were detected at the intersection of the precipitin lines formed from reaction of the antiserum and mitochondrial or supernatant extracts when present in adjacent wells.

Two other approaches were used to investigate the immunological relationship between the faster and slower electrophoretic forms, as cell fractionation provides only an enrichment rather than a discrete separation of the two forms.

Electrophoretic separation of the two major forms of fumarate hydratase present in D98 extracts was effected on cellulose acetate strips in the usual way. This was followed by diffusion against antibody as described in Materials and Methods. The precipitin line detected by enzyme activity stain clearly resulted from interaction of the antiserum with both fast- and slow-migrating forms (see Fig. 4). The lack of intersecting arcs at the junction of the zones of activity again supports the concept that a single class of antibody species reacts equivalently with both enzyme forms. (Note: the weak reaction detected when a hybrid cell extract containing human fumarate hydratase was employed is consistent with the low concentration of human subunits observed in hybrids following normal electrophoresis. The position of the precipitin arc(s) suggests that the antibody reacts with human–mouse hybrid enzyme molecules.) Further confirmation of this immunological pattern was provided by separating the two forms by isoelectric focusing and characterizing their reaction specificity on Ouchterlony plates.

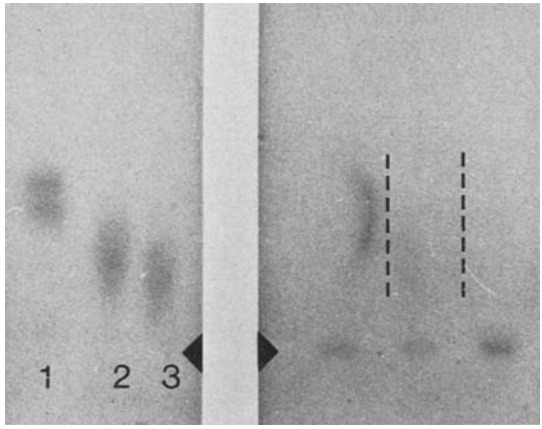

Fig. 4. Immunoelectrophoresis of extracts from human cell line D98 (sample 1), from human–mouse hybrid (sample 2), and from mouse cell line 1R (sample 3). The antiserum was prepared as described in the text. The samples were applied to the left-hand section of a cellulose acetate strip and duplicate samples were applied to the right-hand side. After electrophoresis, the strip was cut into two sections and the left-hand side was immediately stained for fumarate hydratase activity to indicate approximate location of enzyme activity. Antiserum was applied to the duplicate unstained section, as explained in Materials and Methods, at the position indicated by the dashed lines. Note that no reaction was observed with mouse extracts and only faint reaction was detected against the human–mouse hybrid (H22) extract (see text).

Human hearts or placentas were found to be unsuitable sources of material for purification of the two forms of the enzyme. Electrophoresis of extracts from these sources on cellulose acetate strips or isoelectric focusing indicated the presence of additional enzyme activity spread between the expected mitochondrial and cytoplasmic forms. It is not known whether this reflects the aging, or the operation of modification mechanisms not encountered when extracts are prepared from tissue cultured cells. However, small quantities of the two forms of enzyme normally detected in extracts of D98 were prepared by isoelectric focusing. Two peaks of activity (pK 6.7 and 5.8) were found by cellogel electrophoresis to correspond to the slower and faster forms normally observed in whole cell extracts (see Fig. 5). An intermediate peak ($p\mathrm{K} \sim 6.4$) was also resolved by isoelectric focusing and apparently contained the slower-migrating form together with some indistinct faster-

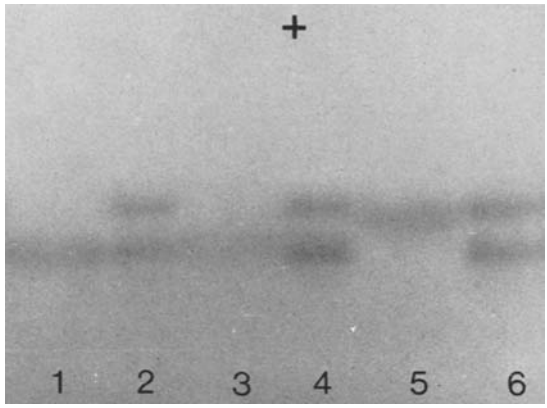

Fig. 5. Separation of the faster and slower electrophoretic forms of fumarate hydratase from an extract of human (D98) cells by preparative isoelectric focusing (see Materials and Methods). Samples in channels 1 and 5 were from the two major peaks of activity resolved after focusing. The *p*K values of these peak fractions were 5.8 and 6.7, respectively. Samples in channels 2, 4, and 6 were of the starting material loaded onto the column. Sample 3 was from an intermediate peak (*p*K 6.4). Enzyme in the peak fractions was precipitated with 65% saturated $(NH_4)_2SO_4$ and suspended in 0.1 ml sodium phosphate buffer (10 mM), *p*H 6.9.

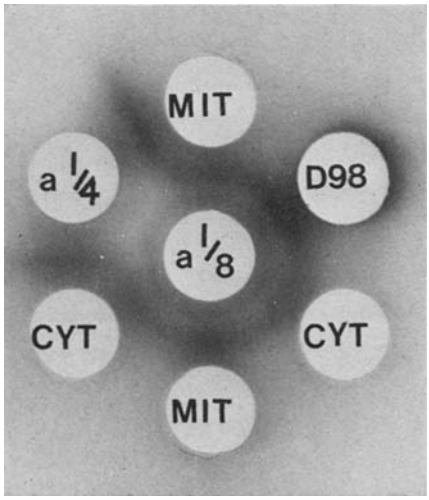

Fig. 6. Ouchterlony diffusion plate demonstrating the complete cross-reaction of mitochondrial and cytoplasmic forms of fumarate hydratase prepared from the human cell line D98 by isoelectric focusing. Each well contained 20 μl of antigen or antiserum. The mitochondrial (MIT) and cytoplasmic (CYT) forms were purified by isoelectric focusing. D98 represents a whole cell extract prepared by sonication of cells in isolation buffer. All samples containing fumarate hydratase were adjusted to about 80 U/ml enzyme activity, "a 1/4" and "a 1/8" represent wells containing one-fourth and one-eighth dilutions of antiserum in phosphate-buffered saline.

migrating material. The two well-separated forms (pK 6.7 and 5.8) were concentrated, adjusted to equal concentration, and examined by Ouchterlony diffusion (see Fig. 6). Both reacted with the antiserum, and no spurs, or discontinuity of any kind, could be detected at the intersection of precipitin line formed from adjacent samples. It seems clear that the antibody employed recognized equivalent determinants on both forms of the enzyme and that antibodies directed against only mitochondrial or only supernatant enzyme determinants were not present.

CONCLUSIONS

Fractionation of human and mouse cultured cells indicated that mitochondria and cytoplasm, respectively, contain predominantly the slower and faster of two electrophoretically distinguishable forms of fumarate hydratase. In addition to the possible involvement of control genes, the data are consistent with the existence of two or more different structural genes, or a combination of one structural gene together with one or more genes determining a modification mechanism.

Analysis of the segregation of human fumarate hydratase in human–mouse hybrids and subclones supports the original assignment of at least one gene responsible for its expression to chromosome 1 (van Someren *et al.*, 1974; see New Haven Conference, 1973). The data presented here represent the detailed enzyme and karyotypic analysis of relatively few well-characterized hybrids and confirms the previously reported localization based on synteny with established chromosome 1 markers.

Furthermore, the results suggest that if more than one structural gene is necessary for the expression of both forms of the enzyme they must be present on the same chromosome, as the presence of both faster and slower human subunits must be invoked to explain the electrophoretic pattern of the hybrids retaining chromosome 1.

Examination of the variation in electrophoretic profile of fumarate hydratase in a variety of cultured animal cell lines provides evidence for a consistent relationship between the two forms. When interspecific differences in electrophoretic mobility are observed, both forms appear to be affected to a comparable extent.

Immunological studies with antisera prepared in mice injected with a commercial preparation of pig fumarate hydratase demonstrate that both human and pig enzyme reacted identically at the limits of resolution of Ouchterlony diffusion plates. Furthermore, both the faster- and slower-migrating forms of the human enzymes were indistinguishable in their behavior with the antiserum. The evidence presented suggests that two electrophoretically distinguishable forms of the enzyme found in cultured

cell extracts possess equivalent immunological determinants. The two enzyme forms could nevertheless represent the products of different structural genes. However, the pattern of variation observed between the various species examined and the similar immunological properties of the two forms lead us to suggest that probably only one structural gene exists, whose product may be modified *in vivo* to an alternative form, and that this modification may be important in determining the final subcellular localization.

It is presumed that if a modification mechanism is present it would be non-species-specific and that the human primary gene product would be modified by the mouse system in hybrid cells. Clearly an unequivocal distinction between the possible explanations awaits either the demonstration of a specific modification system or the elucidation of the primary structure of the two enzyme forms.

Preliminary experiments have thus far failed to show any *in vitro* interconversion between the two enzyme forms by incubation either in the presence of neuraminidase or in the presence of whole cell extracts (human enzyme incubated with mouse cell extract). However, we have noted that each subunit of fumarase is thought to contain three thiol groups (Robinson *et al.*, 1967). It is therefore possible that the diversity of forms may reflect modification to, or interactions of, these groups.

Finally, it appears that human and mouse fumarate hydratase polypeptides can form hybrid molecules *in vivo*. This raises the problem, considering the apparent structural similarity of the mitochondrial and cytoplasmic forms of why intermolecular hybrid molecules are not observed on electrophoresis of human cell extracts. One possibility is that they are present but not detected with the present system. Alternatively, the intracellular localization may be sufficiently effective to prevent extensive interaction.

NOTE ADDED IN PROOF

A report by Meera Khan *et al.* (1974) suggests that possibly two separate loci for guanylate kinase exist which are also situated on chromosome 1.

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