Complementation of gene deletions by cell hybridization

(gene regulation/enzyme expression)

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ABSTRACT Overlapping deletions in chromosome 7 of the mouse are responsible for activity deficiencies of various liver-specific enzymes, including tyrosine aminotransferase (TAT). In an effort to elucidate the nature and type of action of the deleted genes, somatic cell hybridization experiments were carried out. Enzyme-deficient liver cells of homozygous mutant mice or normal liver cells of control newborn mice were hybridized with 2S Faza rat hepatoma cells and the hybrid cell colonies were analyzed for TAT activity. The results show the presence of inducible mouse TAT activity in mutant-2S Faza hybrid cells, thereby excluding the possibility that the structural gene for TAT is included in the gene sequences deleted in the mutants. Furthermore, determinations of mouse glucose-6-phosphate isomerase 1 as a marker eliminate chromosome 7 as the possible carrier of the TAT structural gene, which therefore appears to map on a different chromosome. The deletions interfering with normal enzyme activities apparently include genes other than the respective structural genes, namely those with essential functions in controlling the expression of the differentiated state of the liver cell.

Analytical studies of the role of genes in development and differentiation of biochemical and morphological entities in eukaryotes have been increasingly concerned in recent years with genetic regulatory mechanisms. This trend has been accompanied by a growing awareness of the complexities of organization and interactions of structural and regulatory genes, which are under investigation in various model systems in different organisms. The system under study in our laboratories consists of a series of radiation-induced overlapping deletions in chromosome 7 of the mouse at and around the albino (*c*) locus, all of them lethal when homozygous. The five deletions under study range in size from 1 centimorgan to approximately 5 centimorgans. Complementation tests showed that all of them include deletions of the albino (*c*) locus and four also include deletions of the structural locus *Mod-2* (mitochondrial malic enzyme), which maps 1 centimorgan distal to *c*. The morphogenetic and biochemical effects of the deletions have been analyzed and described in considerable detail (for review, see ref. 1). Morphogenesis of kidney and thymus, ultrastructure of liver and kidney subcellular organelles, embryonic membranes, cleavage of the zygote, activities of several enzymes, and levels of three serum proteins—all these traits are found to be affected in certain homozygotes and double heterozygotes carrying lethal albino deletions.

Biochemical analysis of four affected liver-specific enzymes revealed minute but measurable amounts of residual enzyme activities in lethal albino homozygotes and complete absence of a dosage effect in heterozygotes, which were phenotypically identical with normal homozygotes (2). This, together with the results of morphogenetic and ultrastructural studies, led to the

suggestion that the genes included in the deletions were involved in the regulation of both enzyme activity and induction in cells that expressed the particular enzymes as specific products of differentiation. The respective structural genes coding for the amino acid sequence of the enzymes were assumed to be intact elsewhere in the genome (3). Interaction of different types of genes is obviously essential for normal morphogenesis and biochemical differentiation of the traits affected in the various homozygotes.

In the course of attempts to identify the nature of some of the deleted genes, an experimental approach was designed, based on the elegant system developed by Weiss and her collaborators (4). In particular, these workers found that tyrosine aminotransferase (TAT), a specific differentiation product of liver cells, may be expressed also by nonliver mouse cells—e.g., lymphoid cells—as the result of their fusion to hypertetraploid rat hepatoma cells with rat TAT activity. These results led to the conclusion that diffusible regulatory substances, products of the rat genome, are able to activate the ordinarily silent TAT structural gene in the mouse lymphoid cells. Because our studies provided a strong indication that the deficiency of enzyme activity in lethal albino homozygotes was due to the loss of one or more regulatory genes, we tested the hypothesis that rat hepatoma gene products might be able to replace the regulatory functions missing in the mouse cells and thus activate the intact structural TAT gene mapping elsewhere in the genome of the mutant mouse liver cells. The experiments to be reported here demonstrate the inducibility of mouse TAT activity in hybrid cells derived from mouse liver cells homozygous for one of the deletions, and therefore lacking any inducible TAT activity *in vivo*, after fusion to rat hepatoma cells with high base-line and inducible TAT activity. Thus, missing functions of the deleted mouse genes appear to be complemented by rat genes, the products of which are not species specific and interact with the structural gene of the mouse, resulting in the expression of mouse TAT activity.

MATERIALS AND METHODS

Mutant mice

The strains carrying lethal albino deletions are maintained in our mouse colony. For the experiments to be reported, three of the deletions were used: *c*^{14CoS}, *c*^{3H}, and *c*^{112K}. These are bred as heterozygotes with *c*^{ch} (chinchilla) as the normal allele. Fetuses or newborns homozygous for a deletion are recognized as albinos by their unpigmented eyes, whereas their heterozygous or homozygous normal littermates have pigmented eyes.

Abbreviations: TAT, tyrosine aminotransferase; GPI-1, glucose-6-phosphate isomerase; AECOM, Albert Einstein College of Medicine; MGH, Massachusetts General Hospital.

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Cells, media, and culture conditions

Rat Hepatoma Cells. The rat hepatoma cells, a gift from Mary Weiss, are referred to as 2S Faza. They are hypertetraploid with a mean number of 89 and a range of 77 to 111 chromosomes. They are resistant to 8-azaguanine and 6-thioguanine and sensitive to aminopterin (5).

Primary Mouse Liver Cells. Suspensions from 17- to 18-day-old fetuses were prepared by a modification of the method of Leffert and Paul (6), using collagenase at 3 mg/ml dissolved in Hanks' solution containing Ca^{2+} and Mg^{2+} . Cells from livers of 20-day-old embryos and from newborn mice were obtained by a modification of the method of Amsterdam and Jamieson (7) by digestion with collagenase solution followed by incubation in Hanks' solution containing 2 mM EDTA and dispersion of cells by gentle pipetting. Both methods yielded approximately the same numbers of a mixture of viable epithelioid and fibroblastic cells.

Culture Media. All cells were cultured in McCoy's 5a medium (GIBCO), supplemented with 15% fetal bovine serum, gassed with 10% CO_2 /90% air. Prior to use the 2S Faza cells were maintained in medium containing thioguanine at 3 $\mu\text{g}/\text{ml}$ to select against any possible revertants. Hybrid cell colonies were selected and maintained in full growth medium supplemented with hypoxanthine/aminopterin/thymidine (HAT) (8).

Cell Fusion. Cells were fused in monolayer with polyethylene glycol 1000 (Baker) according to a modification of the method of Pontecorvo (9, 10). About $2-5 \times 10^6$ cells from primary liver cultures were grown for 1-3 days in a 75- cm^2 flask, after which time $4-8 \times 10^6$ 2S Faza cells were added. About 16 hr later this mixed cell culture was washed free of serum with McCoy's 5a medium without serum and exposed for 1 min to 1 ml of 51% (wt/vol) polyethylene glycol in McCoy's 5a. The cells were washed once with medium only and twice with medium containing serum. Subsequently fresh medium was added; it was changed the following day. The cells were then plated in hypoxanthine/aminopterin/thymidine medium at low density to discourage growth of nonfused mouse fibroblasts. Isolated colonies were picked after 4-8 weeks but were not cloned.

Induction of TAT. The cultures were induced by exposing them to medium containing a mixture of 100 nM dexamethasone (Sigma) and 500 nM dibutyryl cyclic AMP (Sigma) for 20-26 hr (11). Cells were stored in liquid nitrogen.

All cell hybrid cultures were prepared and maintained in the laboratory at the Albert Einstein College of Medicine (AECOM). The same culture was divided into two parts for enzyme analysis by the two laboratories. A screening procedure of electrophoresis was used at AECOM and quantitative enzyme analysis was carried out at the Massachusetts General Hospital (MGH), as described below.

Methods used at MGH

Preparation of Cell Extracts. To the pellet of frozen cells was added 2 ml of an extraction buffer containing 0.05 M potassium phosphate at pH 6.6, 1 mM each of EDTA and α -ketoglutarate, and 0.2 mM pyridoxal 5'-phosphate. The contents were sonicated and centrifuged in an SW 50L rotor of a Beckman ultracentrifuge at 45,000 rpm for 1 hr at 3°C. The supernatant was heated at 37°C for 5 min and at 65°C for 6 min. After standing on ice for 30 min, the extract was centrifuged as before and the final supernatant was assayed within a few days.

Determination of Enzyme Activity. Protein was determined by the method of Lowry *et al.* (12). Cytoplasmic TAT activity was determined by the method of Diamondstone (13) as modified by Granner and Tomkins (14) in 1 ml final volume. One nmol of product formed per min at 37°C corresponded to one unit of

TAT activity. The specific activity is expressed in TAT units/mg of protein. Aspartate aminotransferase was determined by the method of Herzfeld and Greengard (15).

Disc Electrophoresis. Samples were electrophoresed in 7.5% polyacrylamide gels 10.5 cm long in a running buffer of 5 mM Tris/NaOH and 38 mM glycine, pH 8.4. The buffer for the cathode chamber was supplemented with 1 mM pyridoxal 5'-phosphate. The sample was diluted to the desired concentration of TAT units with a solution containing 0.0013% bromophenol blue and 13.3% sucrose and run for 1.3 hr at 440-310 V at room temperature. Under these conditions the leading band for rat TAT was close to the anodic end of the gel, followed by that of mouse TAT and, at a considerable distance, by that of cytoplasmic aspartate aminotransferase reacting unspecifically with the moniodotyrosine of the TAT stain. The latter band can be partly suppressed and thereby located by adding 16 mM L-aspartate to the TAT stain. Gels were stained for 1.3 hr at 60°C following the method of Gelehrter *et al.* (16) and Spencer and Gelehrter (17). However, aspartate aminotransferase, as identified enzymatically, turns out not to be present in the extracted bands for rat or mouse TAT.

A more important source of possible interference is the fact that rat TAT from 2S Faza cells, when loaded too heavily, will split into two bands, the second, more cathodic, band assuming a position close to that of mouse TAT. Overloading can be avoided by predetermining for a particular electrophoresis system how many TAT units can safely be loaded without a split—in our case less than 20 units. Furthermore, when there is doubt, extraction of the second band from the gel and reelectrophoresis indicates if this band is due to rat TAT or mouse TAT or a mixture of both. For the definitive identification of mouse TAT in the presence of rat TAT, use was made of the observation of Brown and Weiss (4) that rat TAT is more rapidly inactivated at 75°C than is mouse TAT.

Heat Inactivation of TAT. After electrophoresis the gels were cut lengthwise and one half was stained for TAT activity in order to locate the rat and mouse bands on the other half. Bands were extracted overnight at 3°C in extraction buffer containing bovine serum albumin at 10 mg/ml.

The first-order reaction rate constant (k) of TAT inactivation at 75°C was then determined as follows: Cell extracts were diluted with pH 6.6 extraction buffer containing bovine serum albumin at a final concentration of 10 mg/ml prewarmed for 5 min at 37°C and 2 min at 65°C, and incubated at 75°C for 2, 4, 6, and 8 min. The reaction was stopped on ice. The 2-min sample could serve as the zero time concentration of TAT, because it was ascertained that 75°C was reached in the fluid in the test tubes in less than 2 min. The extracts of bands 1 and 2 were always run simultaneously and in close proximity in the same water bath. The samples were then analyzed for TAT and the logarithm of concentration was plotted against time.

In order to minimize the possible effect of slight fluctuations in the water bath temperature, calculations of temperature inactivation were based on the ratio k_1/k_2 rather than on individual values of the rate constants for rat and mouse. These calculations originate from the observation that, in mixtures of rat and mouse TAT, the most anodic electrophoretic band contains rat TAT only, while the closely following second band generally contains a mixture of mouse and rat TAT, because it was necessary to load the gels with about 30-50 TAT units so as to obtain enough material for analysis. A second observation is that, when rat and mouse TAT are electrophoresed on separate gels and extracted, the ratio k_1/k_2 at 75°C is close to 2.0. This was also the case when the ratio was determined without prior electrophoresis. This leads to the following equation: $2/(2x + y) = (k_1/k_2)$ as determined, in which x and y are the fractional concen-

FIG. 1. Slab gel electrophoresis of TAT. Between 16 and 20 units of TAT activity were loaded in each slot. Slot 1, artificial mixture of rat and mouse lysates, showing rat (R) and mouse (M) bands only. Slot 2, lysate of hybrid cell colony, showing rat, hybrid (H), and mouse bands. Slot 3, lysate of hybrid cell colony showing rat band only. Slot 4, lysate of 2S Faza rat hepatoma cells. Slot 5, lysate of adult mouse liver.

trations of rat and mouse TAT, respectively, in the extract of the second band. Transposing, one obtains the value of $(2x + y)$, which allows y to be calculated or to be read from a graph of percent mouse TAT against a scale ranging from 1 to 2, 1 being 100% and 2 being 0% mouse TAT. Control experiments using known mixtures of mouse and rat TAT ranging between 15% and 75% mouse TAT gave satisfactory recoveries.

Methods used at AECOM

The extraction buffer contained 0.1 M potassium phosphate at pH 7.3, otherwise the method of preparing cell extracts was the same in the two laboratories. The method for TAT determination and the definitions of units and specific activity were also the same.

Slab Gel Electrophoresis. The separation gels were of the same composition as those used for disc electrophoresis. In addition, a stacking gel containing 4% polyacrylamide in 12.5 mM Tris-HCl, pH 7.3, was used. The running buffers and the pH were also the same. The samples were diluted to 160 TAT units/ml with extraction buffer containing 0.0025% bromphenol blue and 5% sucrose final concentration. For each sample, 8 and 16 units of TAT activity were loaded on the gel. The run was about 3/4 hr at 160 V through the stacking gel and at 320 V for 1 hr through the running gel. The gels were stained for TAT for about 45 min at 60°C according to the method of Gelehrter *et al.* (16), with the following modifications: the gels were not presoaked in ferricyanide and the buffer was 50 mM potassium phosphate, pH 7.6. Aspartate aminotransferase activity, visualized by using two different methods of staining (18), was shown to be located

in a different position on the gel from TAT activity. This result coincides with that given in a previous section.

Glucose-6-Phosphate Isomerase (GPI-1) as Marker for Chromosome 7. Presence or absence of chromosome 7 carrying the deletions was determined in the cell cultures with the help of the enzyme GPI-1 as a marker because its structural locus maps on this chromosome. The electrophoretic identification of rat and mouse GPI-1 was carried out with the Titan III cellulose acetate method of Eicher and Washburn (19). With this method rat, mouse, and hybrid bands can be clearly distinguished.

RESULTS

A total of 12 fusions between mutant mouse liver and 2S Faza rat hepatoma cells were the source of 57 colonies, 52 of which were used for enzyme analysis. Seventeen fusions of normal littermate liver and 2S Faza rat hepatoma cells provided the control material. Altogether 77 cultures were derived from these fusions and 72 of the cultures were analyzed for enzyme activity.

Subsequent comparison and repetition of TAT analyses carried out at MGH and AECOM resulted in complete agreement between results obtained with the different methods in the two laboratories.

Liver cells of both mutant and control newborn mice fail to show significant base-line or induced TAT activity in primary cultures, nor have they regained the ability to express TAT at the time of fusion. 2S Faza rat hepatoma cells, on the other hand, have appreciable base-line TAT activity, which is further inducible by steroids, at the time of hybridization. Fig. 1 is a photograph of slab gel electrophoresis of mouse and rat TAT. Slots 2 and 3 represent two different cell hybrid colonies: slot 2 shows rat, mouse and presumed rat/mouse heterodimer bands, slot 3 shows the rat band only. Slot 1 contains an artificial mixture of 30% mouse and 70% rat TAT; such mixtures never exhibited a heterodimer band.

Table 1 summarizes the results of the analyses of mouse TAT activity. The percentages of colonies with and without mouse TAT activity are the same in hybrids derived from mutant as those derived from control mouse liver cells. Each fusion experiment produced mouse TAT positive, negative, and inconclusive colonies. Each colony is therefore considered independently. Results were termed "+" if the proportion of mouse TAT to total TAT activity was 10% or more as measured with the disc gel and heat inactivation methods and when strong hybrid heterodimer as well as homodimer mouse bands appeared in the slab gels. Inconclusive results ("P") were those in which slab gel electrophoresis only was used and mouse and heterodimer hybrid bands were faint. The proportions of hybrid colonies expressing inducible mouse TAT activity were 25% in hybrid cells with a mutant mouse parent as well as in those with a control mouse parent. Seventy-five percent of all mutant and control hybrid colonies, counting the inconclusives as negative, failed to be inducible for mouse TAT.

In Table 2 is listed a smaller series of experiments in which the methods used at MGH (as described above) permitted

Table 1. Summary of results of TAT analysis in cell hybrid colonies by slab gel electrophoresis

Type of fusion	No. of fusions	No. of colonies derived	Colonies with inducible mouse TAT activity (+)		Colonies lacking inducible mouse TAT activity (-)		Colonies with inconclusive results (?)		Total no. of colonies analyzed
			No.	%	No.	%	No.	%	
Mutant mouse liver and 2S Faza rat hepatoma	12	57	14	27	27	52	11	21	52
Normal mouse liver and 2S Faza rat hepatoma.	17	77	18	25	39	54	15	21	72

Table 2. Summary of results of TAT analysis in hybrid cell colonies

Type of hybrid	No. of Exps.	Specific activity, nmol/mg protein per min	% distribution of TAT activity		Ratio of <i>ks</i> , band 1/band 2	Mouse TAT in % of total TAT	
			Band 1	Band 2		Range	Mean
Mutant mouse liver with 2S Faza rat hepatoma	7	1540	52.9	47.1	1.34	17-33	23.8
	4	1710	61.7	38.3	1.0	0-7	1.7
Control mouse liver with 2S Faza rat hepatoma	8	1100	57.7	42.3	1.40	10-38	24.1
	8	1490	68.6	31.4	1.07	0-8	3.9

Results are means for the indicated numbers of experiments. The first-order rate constant (*k*) for enzyme inactivation was determined at 75°C. All cells were induced for TAT with dexamethasone/cyclic AMP.

mouse and rat TAT activity to be expressed quantitatively. The same hybrid colonies were also examined in the AECOM laboratory by slab electrophoresis, and this was conducted as a blind study. The agreement with respect to the presence or absence of mouse TAT in a given hybrid cell colony was excellent, there being only three discrepancies, which were resolved subsequently by examination of new subcultures (grown from the original frozen-stored hybrid cell colonies). As indicated before, the cut-off point chosen in Table 2, below which the presence of mouse TAT seemed doubtful, was 10%, and this agreed well with the results of electrophoresis. The table shows the range as well as the average value for percent of induced mouse TAT in the hybrid colonies. These as well as other characteristics measured show close correspondence between the mutant and control hybrid series.

Table 3 presents the results of tests of mutant and control hybrid colonies for mouse GPI-1 (the marker for chromosome 7) and its association with the expression of mouse TAT. The distribution of the concordant and discordant colonies suggests independent assortment of the two enzymes in the case of the mutant hybrid cell colonies. Thus, chromosome 7 does not appear to carry the structural gene for TAT, and is not required for this enzyme to be expressed under the present experimental conditions. Similarly, the results of the control hybrid cell colonies exclude chromosome 7 as the site of the structural gene for TAT.

DISCUSSION

Liver cells of newborn mice homozygous for a deletion in chromosome 7 and deficient in TAT activity as well as inducibility for this enzyme can be caused to express inducible mouse-specific TAT activity when fused to heteroploid 2S Faza rat hepatoma cells. Obviously such a result is possible only if the structural gene for TAT is intact in the mutant mouse cells. Furthermore, whereas a heteropolymer band was never observed upon elec-

trophoresis of artificial mixtures of mouse and rat TAT, hybrid cells expressing mouse TAT did produce such a band. Presumably, the dimer proteins of mouse and rat TAT (20) are stable, and heterodimers can be formed only when mouse and rat monomers are produced and present simultaneously in the hybrid cell. The existence of a mouse/rat heterodimer molecule presupposes the availability of a mouse TAT monomer and thus demonstrates the expression of the mouse TAT structural gene in the hybrid cell. This gene can therefore not be part of the sequences deleted in chromosome 7 of the deletion homozygotes. If the TAT structural gene maps outside the deleted portion of the genome, the reason for the enzyme defect of homozygotes must be the absence of other essential genes included in the deletions and having regulatory functions. The use of the enzyme GPI-1 as a marker for chromosome 7 served to exclude this chromosome with great likelihood as a possible site for the TAT structural gene. The actual and direct identification of the chromosome on which the TAT structural gene maps has not yet been made. The fact that identical proportions of mutant and control mouse cell hybrid colonies express inducible mouse TAT activity or fail to do so demonstrates strikingly that presence or absence of genes included in the deletions does not determine the ability of the mouse/rat hybrid cells to express this particular function. The reexpression in control cell hybrids of TAT activity extinguished in primary control liver cell cultures indicates that even the genetically normal liver cell requires the activating stimulus provided by the rat genome in order to revert from its dedifferentiated state in the primary culture to reexpression of its tissue-specific enzyme TAT.

In the interpretation of the cell hybridization experiments we are concerned with the possible reflection of the results on the genetic control of normal liver cell differentiation, with the liver-specific enzyme TAT serving as marker. Analysis of the somatic cell hybrids demonstrates clearly that normally the gene sequences near the albino (*c*) locus included in the deletions must play a decisive role in promoting the differentiated state of the liver cell and in enabling it to express certain of its differentiated functions. These regulatory genes do not function in nonliver cells—i.e., their active state is restricted to cells with liver- (and possibly kidney-) specific functions. Associated with the control of differentiation appears to be the control of enzyme induction by these genes, because their deletion from the genome is correlated not only with a severe decline of the base level of TAT activity but also with total absence of inducibility *in vivo*. At this time, it is not possible to venture a guess as to the specific step(s) in the induction mechanism controlled by these regulatory genes. It will be interesting to see if the deficiencies of other enzymes affected by the deletions, particularly glucose-6-phosphatase and serine dehydratase, also can be corrected by hybridization with 2S Faza rat hepatoma cells. This would indi-

Table 3. Association of expression of mouse TAT and GPI-1 as marker of chromosome 7

Type of hybrid	No. of colonies				Total
	TAT ⁺ GPI ⁺	TAT ⁻ GPI ⁻	TAT ⁻ GPI ⁺	TAT ⁺ GPI ⁻	
Mutant mouse liver and 2S Faza rat hepatoma	7	6	11	2	26
Control mouse liver and 2S Faza rat hepatoma	11	10	3	6	30
Total	18	16	14	8	56

cate that a group of liver-specific enzymes might be coordinately regulated by the gene sequences identified by these deletions. The demonstration of a series of genes concerned with the promotion and control of a tissue-specific state of differentiation offers a potential for the study of structure, organization, and function of eukaryotic regulatory genes.

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