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REGULATION OF EXPRESSION OF TYROSINE AMINOTRANSFERASE IN SOMATIC CELL HYBRIDS BETWEEN RAT HEPATOMA CELLS AND MOUSE FIBROBLASTS

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Summary: Somatic cell hybrids between rat hepatoma cells and mouse 3T3 fibroblasts fail to produce the liver-specific enzyme tyrosine aminotransferase. A novel approach using gamma-irradiation to induce chromosome loss from the non-expressing parent cell was applied to dissect genetically the factors in 3T3 cells that interact with the regulation of expression of tyrosine aminotransferase in these hybrids. Suppression of basal and steroid-inducible tyrosine aminotransferase activities was progressively relieved with increasing dose of radiation. The wide range in degree of reexpression suggests a complex of regulatory mechanisms. Suppression of steroid-inducibility was not linked to the mouse X-chromosome. Nor did the mouse genome affect the modulation of enzyme activity induced by insulin and by serum.

Hybrids between cells expressing a tissue-specific protein and cells of a different kind most often fail to produce the given protein, but "household" proteins specified by both parents continue to be synthesized (for a review, see (1)). Occasionally, tissue-specific proteins are reexpressed in such hybrids, although the level of synthesis of the given protein rarely reaches that in the parental cell (e.g. 2,3,4). The fact that reexpression occurs in hybrids from which chromosomes, presumably from the non-expressing parent cell, have been lost indicates that the maintenance of suppression requires the continuous presence of some suppressing factor(s) contributed by the non-expressing parent cell (1).

Since spontaneous chromosome segregation is slow and random, and may be of even undesired origin, we applied a new approach to study gene regulation by using gamma-irradiation to induce chromosome loss from the non-expressing parent cells (5,6,7). Within a limited period of time large numbers of hybrids can be obtained in which the chromosomal contribution from the irradiated partner ranges from the entire genome to practically only one gene, i.e. the

[1] Abbreviations: TAT, tyrosine aminotransferase; HTC, hepatoma tissue culture cells; HPRT, hypoxanthine phosphoribosyltransferase; IDH, isocitrate dehydrogenase NADP; LDH, lactate dehydrogenase; GPI, glucosephosphate isomerase; G6PD, glucose-6-phosphate dehydrogenase.

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one for which the hybrids are selected (7,8,9). The technique was applied to study regulation of expression of the liver-specific enzyme tyrosine aminotransferase (TAT[1]) (E.C. 2.6.1.5) in hybrids between rat hepatoma cells (HTC) and mouse 3T3 fibroblasts. HTC cells produce low levels of TAT that can be induced up to 15-fold by glucocorticoids (10). This seems to be the first attempt to a systematic genetic dissection of factors in the non-expressing partner cell that interact with the production of a tissue-specific protein specified by the other partner cell.

METHODS

Cells: HTC TG30 is deficient of hypoxanthine phosphoribosyltransferase (E.C. 2.4.2.8) (11). It is a clonal derivative of the rat hepatoma cell line HTC (10). The chromosome mode is 56, and the range is 54-59.

3T3 TK^- is a thymidine kinase-deficient (E.C. 2.7.1.21) mouse fibroblast-like cell. The chromosome mode is 66, the range 64-69. The detailed history of the cell line is unknown.

All cells were grown in monolayer cultures. Hybrids were maintained in medium supplemented with hypoxanthine (0.1 mM), aminopterin (0.01 mM), and thymidine (0.04 mM) (12) unless stated otherwise. Cells in their logarithmic phase of growth were harvested by trypsinization and washed once in phosphate buffered saline (Na_2HPO_4, 6.5 mM; KH_2PO_4, 1.5 mM; NaCl, 136.9 mM; KCl, 2.7 mM; pH 7.3) before further processing. Procedures for irradiation of cells, cell fusion, selection of hybrids, and chromosome analysis are described in (13). Only one clone was isolated from each flask to ensure that all clones studied were of independent origin.

TAT assay: Cells were incubated as described in the legends to the figures. Cells resuspended in 100 µl buffer, consisting of KH_2PO_4, 29 mM; Na_2HPO_4, 71 mM; pyridoxal phosphate, 0.1 mM; α-ketoglutaric acid, 1.0 mM; pH 7.3 (14), were lysed by rapid freezing and thawing, and the debris was pelleted in a Beckman Airfuge at 100,000 x g for 15 min at 4 °C. The supernatant was tested for TAT specific activity by the modification (14) of the method of Diamandstone (15). One milliunit of enzyme catalyzes the formation of 1 nmole p-hydroxyphenylpyruvate per min at 37 °C. Enzyme specific activity is expressed as milliunits per mg protein. Immunotitration of TAT was done with antiserum, produced in sheep, to highly purified rat liver TAT. An appropriate amount of antiserum mixed with the sample was incubated for 2 h at 37 °C and subsequently for 16 h at 4 °C. Protein concentrations were determined by the method of Lowry et al. (16). To ensure that the enzyme activities determined were comparable between the groups, the parental line was identically analysed along with each group.

Electrophoretic separation of mouse and rat isozymes was done on Cellogel (Chemetron, Milan). Isocitrate dehydrogenase NADP (E.C. 1.1.1.42) and glucosephosphate isomerase (E.C. 5.3.1.9), respectively, were separated in buffer consisting of Tris (33.8 mM), EDTA (4 mM), citric acid (6.3 mM), pH 7.5 (17), for 4 h at 200 V, and glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) and lactate dehydrogenase (E.C.1.1.1.27), respectively, in Tris-glycine (52 mM) buffer, pH 8.8, for 3 h at 200 V. Bands of isozymes were developed according to the techniques described in (17) and (18). Electrophoretic separation of mouse and rat HPRT was done as previously described (13).

Materials: Insulin (Monotard[R]) was a gift from NOVO Ltd., Copenhagen. TAT antiserum was kindly provided by Dr. D.W. Martin,Jr., San Fransisco. Dexamethasone acetate was from Sigma. Cells were grown in Eagle's minimum essential medium (GIBCO, Scotland) supplemented with 10% foetal calf serum (Sera-Lab, Sussex, UK).

Table 1. Frequency of retention of mouse marker enzymes in hybrid clones between HTC TG30 and gamma-irradiated mouse 3T3 cells. Figures in parentheses give the number of clones examined.

Jkg^{-1}	Enzyme				
	IDH	LDH	GPI	retention of all autosomal enzymes	G6PD
0 (10)	1.0	1.0	1.0	1.0	1.0
10 (5)	1.0	1.0	1.0	1.0	1.0
20 (10)	0.8	0.8	0.9	0.8	0.8
30 (7)	0.6	0.3	0.6	0.4	0.7
40 (6)	0.3	0.2	0.3	0.0	0.5
50 (6)	0.2	0.2	0.3	0.0	0.3
100 (8)	0.0	0.0	0.0	0.0	0.0

RESULTS

Retention of mouse chromosomal material: C-banding of metaphase chromosomes with the fluorescent dye Hoechst 33248 shows that hybrids between HTC TG30 and intact 3T3 TK^- cells have chromosome complements very close to what would be expected from the fusion of two modal parental cells (data not presented). Since gamma-irradiation causes fragmentation of the mouse chromosomes, thus making their identification difficult, we chose to monitor retention of mouse chromosomal material by screening the hybrids electrophoretically for expression of selected autosomally and X-chromosomally specified enzymes. Table 1 shows that retention frequencies are greatly reduced by irradiation and that this effect is clearly dependent on the dose of gamma-irradiation given.

Inducibility with dexamethasone: The shaded areas in Figure 1 represent the range of basal and steroid-induced TAT activities, respectively, in 29 independent subclones of HTC TG30. On the basis of this analysis the cell line is considered homogeneous in terms of production of TAT. Thus the possibility of selecting a hybrid in which the parental hepatoma cell lacks TAT activity in the first place seems remote. 3T3 TK^- does not produce TAT and is not inducible.

Prior to fusion with HTC TG30, 3T3 TK^- cells were given doses of gamma-irradiation between 0 and 100 Jkg^{-1}. HTC TG30 is deficient of HPRT for which the hybrids were selected. Therefore, the minimal contribution from the 3T3 parent cell is the X-linked gene coding for HPRT. At passage 2-4, i.e. 3-4 weeks after isolation, hybrid cells were analysed as described in the legend

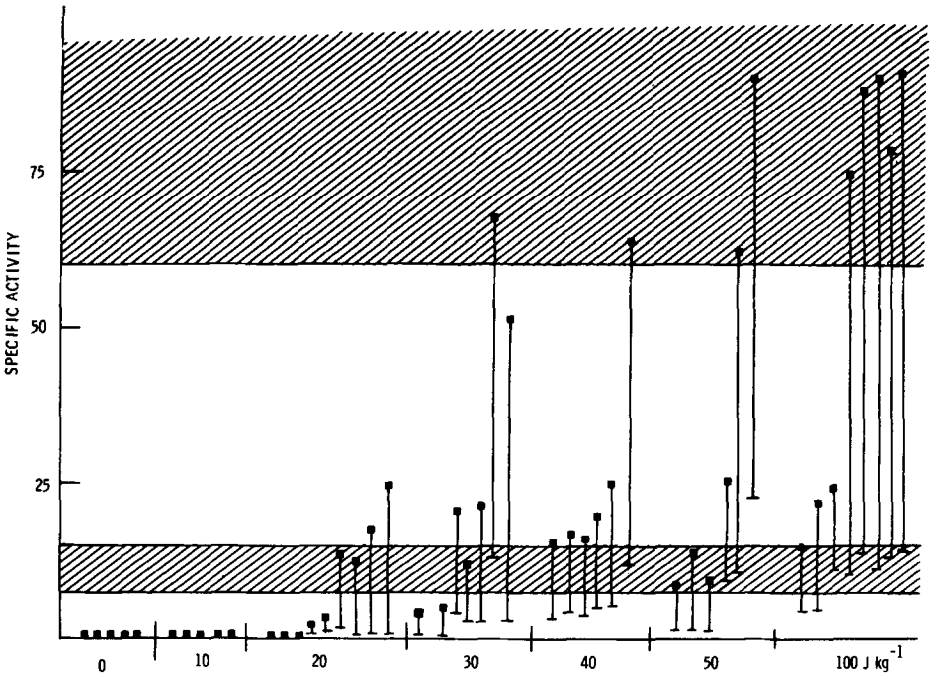

Figure 1. Specific basal (-) and steroid-induced (■) TAT activities (mU) in hybrid clones of HTC TG30 and irradiated or non-irradiated 3T3 TK⁻. 2.4 x 10^6 cells were incubated for 20 h in medium (10% foetal calf serum) supplemented with or without 10^{-6} M dexamethasone acetate. The shaded areas represent basal and induced levels of parental TAT.

to Figure 1. Neither basal nor steroid-induced TAT activities were detected in hybrid clones from classes of 0 and 10 Jkg^{-1} of irradiation, but at doses of 20 Jkg^{-1} or more suppression of both basal and steroid-inducible TAT was progressively relieved, showing a wide range in the degree of reexpression. However, only in the 100 Jkg^{-1} class more than half of the hybrids had activities of TAT within the range given by the HTC TG30 parent cells. Reexpression was always preceeded by that of inducible TAT, and none of the hybrids had high levels of basal TAT activity in the absence of induced activity.

Little mouse chromosomal material is retained in the hybrids with heavily irradiated 3T3 cells (Table 1). Nevertheless, TAT levels are reduced in some of these hybrids (Figure 1). It is, however, unlikely that such hybrids originate from fusions with low-expressing HTC cells in the view of the analysis shown in Figure 1. Since it has been suggested that suppression of induction of TAT in hybrids between rat hepatoma cells and diploid human fibroblasts is regulated by a gene on the human X-chromosome (4), and since our hybrids were selected for retention of the X-linked mouse HPRT, there remains

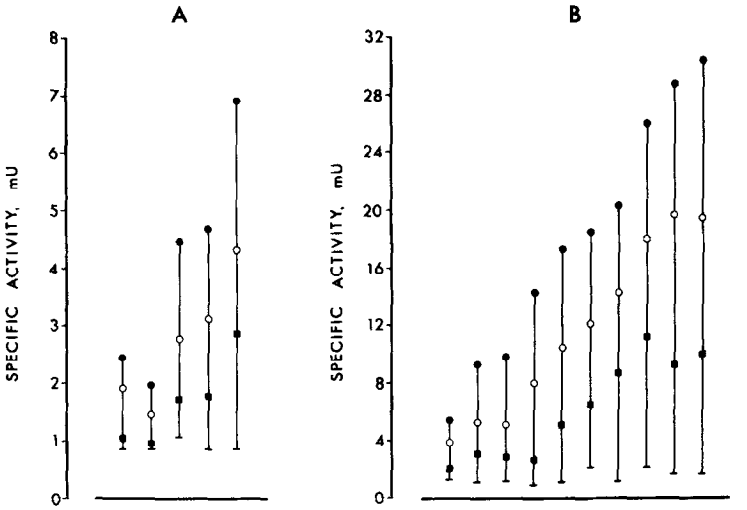

Figure 2. Insulin and serum responsiveness in hybrid clones of HTC TG30 and irradiated 3T3 TK$^-$ cells; (A) 10 Jkg^{-1}, (B) 20 Jkg^{-1}. 2.4 x 10^6 cells were incubated in serum-free medium supplemented with (■) or without (-) 10^{-6} M dexamethasone acetate. After 18 h, 100 mU/ml insulin (o), or 100 mU/ml insulin + 10% foetal calf serum (●) was added to the flasks containing dexamethasone medium, and the cells were incubated for additional 2½ h.

the possibility that also the mouse X-chromosome contains a gene that controls the regulation of steroid-induction of TAT.

To test this possibility, 3 clones from each of the 50 and 100 Jkg^{-1} classes were back-selected in medium supplemented with thioguanine (50 µg/ml), in which only cells deficient of HPRT can grow. Before back-selection, all hybrids produced the mouse HPRT enzyme. No other marker enzymes were detected in the 100 Jkg^{-1} hybrids, but 2 of the 50 Jkg^{-1} hybrids expressed in addition to the mouse X-linked G6PD either LDH or GPI. After back-selection, none of the X-chromosomally specified enzymes were detected in the 6 hybrids, but the mouse LDH and GPI enzymes, respectively, were still produced in the 50 Jkg^{-1} hybrids. Full inducibility of TAT was not restored in any of the 6 hybrids (data not shown).

Inducibility with insulin and serum: Addition of insulin or dialyzed serum to HTC cells previously incubated with dexamethasone in serum-free medium causes a further doubling in TAT activity (19,20). When serum and insulin are added simultaneously, the increase in TAT activity is approximately the sum of the separate effects of the two. This suggests that serum and insulin affect different aspects of regulation of TAT (21).

We have examined whether these two mechanisms of regulation are suppressed in hybrids, and if so, whether they can be reexpressed independently. Hybrids at passage 7-9 were incubated as described in the legend to Figure 2. TAT activities increased approximately 2-fold in all irradiated hybrids examined and in one of 10 hybrids with intact 3T3, when cells were incubated in medium containing serum (data not shown) or insulin; the increase was 3-4-fold when the compounds were added simultaneously. In no case was the effect of insulin expressed in the absence of serum, or vice versa. The authenticity of TAT in the low-expressing hybrids was verified by immuno-precipitation with specific antiserum to rat liver TAT (data not shown). 3T3 cells did not respond to serum and/or insulin. It is noted that some of the TAT-producing hybrids (Figure 2) were previously classified as non-expressing. The reason for this discrepancy may be ascribed loss of chromosomal material from the 3T3 cell during the course of continued cultivation.

DISCUSSION

We have examined a large number of rat hepatoma hybrids in which the chromosomal contributions from the non-expressing 3T3 partner cell ranged from the entire genome to only a small amount of cryptic chromosomal material. In early passages, hybrids with intact 3T3 cells failed to produce TAT, but concomitant with increasing loss of mouse chromosomal material, suppression was progressively relieved. This shows that suppressing elements in 3T3 cells are radiation-sensitive, but also that suppression is not an all-or-none phenomenon. Apparently very little 3T3 chromosomal material needs to be lost in order to relieve suppression (cf. Figure 2), but the frequencies of fully expressing hybrids suggest the total elimination of suppressing elements in 3T3 cells in order to produce fully expressing hybrids. This is in good agreement with previous observations that reexpression is most often partial only, but, on the other hand, is correlated with only limited loss of chromosomes from the non-expressing parent cell (1).

Regulation of suppression of steroid-inducibility was not linked to the mouse X-chromosome. Nor was modulation by serum and insulin of steroid-induced TAT levels affected by the 3T3 genome. Since the effect of insulin is caused by a selective inhibition of degradation of TAT (22), our findings suggest that regulation of expression in the above hybrids is at a level more distant to that of posttranslation.

The wide range in the degree of relief of suppression indicates more complex mechanisms of regulation than existing models suggest (23). We believe that most, if not all, of the suppressing factors in 3T3 cells are

of chromosomal origin, but there is some evidence that in somatic cell hybrids extra-chromosomal elements affect expression of differentiated functions (24) and of malignancy (8). This possibility is presently being investigated in the above hybrids.

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