Tyrosine Aminotransferase Gene Expression in a Temperature-sensitive Adult Rat Liver Cell Line

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ABSTRACT

Regulation of tyrosine aminotransferase gene expression was studied in an adult rat hepatocyte line (RALA255-10G) which is temperature sensitive for the maintenance of the differentiated liver phenotype. Glucocorticoid hormones such as cortisol were necessary for expression of the aminotransferase gene. In the absence of these steroids, enzyme synthesis, activity, and mRNA accumulation were virtually abolished. In the presence of cortisol at 33°C, RALA255-10G cells showed characteristics of malignant transformation and contained little tyrosine aminotransferase activity, synthesized low levels of this enzyme, and produced low levels of enzyme mRNA. At 40°C, cells maintained in the presence of cortisol regained the normal, differentiated phenotype, and tyrosine aminotransferase activity increased only for the first 2 days, probably due to thermal inactivation of this enzyme at 40°C. Dibutyryl cAMP alone was not sufficient to induce expression of the tyrosine aminotransferase gene, but it enhanced the induction caused by cortisol. Immunocytochemical studies revealed that the enhanced expression of the tyrosine aminotransferase gene at 40°C and in the presence of cortisol or cortisol plus dibutyryl cAMP resulted from an increase in both the number of cells producing this enzyme and the quantity of tyrosine aminotransferase synthesized per cell.

INTRODUCTION

The use of virus in studies of cell differentiation and transformation has provided much information about the regulation of cell-specific gene expression during development and carcinogenesis. In several cell lineages such as muscle (1), cartilage (2), and pigment cells (3) it has been shown that viral transformation prevents initiation of the expression of genes which are characteristic of the respective lineages. In these instances the data suggest that differentiation of the precursor cell has been prevented; hence myogenesis, chondrogenesis, or melanogenesis has been blocked. In all of these studies, a temperature-sensitive mutant of Rous sarcoma virus was utilized, thus allowing for a study of expression in either permissive or nonpermissive conditions for the virus.

The isolation of tsA4 mutants of SV40, temperature sensitive in the gene required for maintenance of transformation, has led to the transformation and isolation of placental (4) and liver (5, 6) cell lines which are temperature sensitive for growth and differentiation. It is possible to culture these SV40 tsA4 mutant-transformed cells at a permissive temperature in order to obtain a large number of cells. Subsequently they can be shifted to a nonpermissive temperature to enable changes in expression of tissue-specific genes to be studied. Using the SV40 tsA255-transformed adult rat liver cell line RALA255-10G (5), we have shown that these cells synthesize low levels of albumin and transferrin at the permissive temperature. At the nonpermissive temperature, synthesis of both albumin and transferrin is markedly enhanced. These results suggest that the expression of these liver-specific genes is affected by the state of the cell with respect to transformation, and furthermore, that the transformed state leads to suppression of the expression of these two liver-specific markers.

In this study, the expression of the tyrosine aminotransferase gene is studied in RALA255-10G cells. Tyrosine aminotransferase is a liver-specific enzyme which is normally detected after birth (7). The hormonal control of tyrosine aminotransferase has been one of the most intensively studied phenomena in mammalian cell regulation (8, 9). Glucocorticoids increase enzyme activity and synthesis due to an increase in the amount of translatable mRNA (10, 11). In addition, tyrosine aminotransferase activity is increased by cAMP in vivo (12) and in cultured hepatocytes in the presence of glucocorticoid (13). In this report, we show that expression of the tyrosine aminotransferase gene in RALA255-10G cells is temperature sensitive and dependent upon the presence of glucocorticoid hormone. Bt2cAMP alone cannot induce expression of the tyrosine aminotransferase gene. However, in the presence of glucocorticoid, Bt2cAMP can enhance the induction by glucocorticoid.

MATERIALS AND METHODS

Cell Culture. The SV40 tsA255 mutant virus-transformed rat adult liver cell line RALA255-10G (5) was grown in a-modified minimal essential medium supplemented with streptomycin (100 μg/ml), penicillin (100 units/ml), 4% fetal bovine serum, and 1 μM cortisol. Initially, cells were grown at 33°C (permissive temperature). After 3 to 5 days of growth at 33°C (Day 0), some of the cultures were shifted to 40°C (nonpermissive temperature). The medium was changed every 2 days.

Tyrosine Aminotransferase Assay. Tyrosine aminotransferase was assayed using a radiochemical method described previously (14). Enzyme activity was determined in fractions eluted from a carboxymethyl-Sephadex column to which extracts from cultured hepatocytes were applied. Activity is expressed as μmol/h/mg protein. Reaction blanks were incorporated for each determination. The limit of sensitivity of the assay is set by the range in dpm of the blanks (40 units/mg protein).

Biosynthesis of Tyrosine Aminotransferase. Cultures were labeled for 3 h at 33°C or 40°C by incubation in methionine-free medium to which L-[35]S-methionine at 100 μCi/ml (New England Nuclear) was added (5). Cell lysates in Buffer A (PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 500 μg trypsin inhibitor) were prepared as described previously (5). Polypeptide immunoprecipitated by rabbit antiserum to rat tyrosine aminotransferase (anti-tyrosine aminotransferase-precipitable polypeptide) was isolated by a double antibody method. Samples of cell lysates were cleared of nonspecific radioactive contaminants by adding 0.1 ml of Pansorbin (10% in Buffer A), mixing for 3 h, then centrifuging at 5000 rpm for 5 min in a Beckman microfuge. Antiserum to tyrosine aminotransferase was added to the supernatant, and the mixtures were incubated overnight at 4°C. Tyrosine aminotransferase was isolated by precipitation with Protein A.
A-Sepharose. The immunoprecipitates were sedimented, washed sequentially with Buffer A, Buffer A containing 1 M KCl, and PBS, and electrophoresed in a 10% polyacrylamide-SDS slab gel (15). Radioactivity was visualized by fluorography (16). Apparent molecular weights were determined using the following [14C]methionine-labeled protein standards obtained from Amersham: myosin (M, 200,000), phosphorylase B (M, 92,500), bovine serum albumin (M, 69,000), ovalbumin (M, 46,000), and carbonic anhydrase (M, 300,000).

RNA Electrophoresis, Blotting, and Hybridization. Total cell RNA was extracted by the guanidiinium thiocyanate method of Chirgwin et al. (17). RALA255-10G cells were lysed with buffer containing 4 M guanidinium thiocyanate, and RNA in the cell lysates was isolated by sedimentation through cesium chloride. Poly(A) + RNA was obtained by subjecting the total RNA to oligothymidylic acid-cellulose affinity chromatography (18).

Poly(A) + RNAs were resolved on 1.2% agarose gels in the presence of formaldehyde (19). RNA samples were dissolved in buffer (0.2 M morpholinopropanesulfonic acid, pH 7.0, 50 mM sodium acetate, and 1 mM EDTA, pH 8.0) containing 50% formamide and 2.2 M formaldehyde, heated for 10 min at 65°C, and electrophoresed on a 1.2% agarose gel in 2.2 M formaldehyde with 0.2 M morpholinopropanesulfonic acid, pH 7.0, 50 mM sodium acetate, and 1 mM EDTA, pH 8.0, as circulating electrode buffer.

Denatured RNAs were transferred from agarose gel to Zetabind membranes (AMF Cuno, Microfiltration Production Division, Meriden, CT) using the Electro-Blot procedures. Nick-translated rat tyrosine aminotransferase complementary DNA fragment, pcTAT-3 (20), and rat β-actin fragment were used as hybridization probes. Hybridization in the presence of dextran sulfate and washing was performed as described by Wahl et al. (21) with the following modifications. RNA blots were washed in 0.3 M sodium chloride-0.03 M sodium citrate, pH 7.2, containing 0.1% SDS for 30 min each at room temperature, and then 4 times in 0.015 M sodium chloride-0.0015 M sodium citrate, pH 7.2, containing 0.1% SDS for 60 min each at 68°C.

Immunocytochemistry. Cultures were washed 3 times with Hanks' balanced salts solution and then fixed for 5 min at 4°C with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.45. The fixed cultures were then washed twice with ice-cold PBS and stored at 4°C in 0.1 M cacodylate buffer for up to 1 wk before immunolocalization of tyrosine aminotransferase was performed. To localize tyrosine aminotransferase, samples were incubated with 0.1 M lysine in PBS/saponin for 15 min followed by a 60-min incubation in 10% fetal calf serum in PBS/saponin at room temperature. This was then followed by incubation with rabbit anti-tyrosine aminotransferase serum (1:100 or 1:200 dilution) in PBS/saponin for 60 min. The samples were then washed 3 times with PBS/saponin and incubated with peroxidase-coupled goat anti-rabbit IgG diluted 1:1000. After this, the unbound second antibody was washed off using three changes of PBS. The bound antibody was then visualized by incubation with diaminobenzidine (0.05% in 0.05 M Tris-HCl, pH 7.5) in the presence of 0.3% H2O2 for 20 min at room temperature. The preparation was finally washed 3 times with PBS and mounted in Kaiser's glycerol/gelatin (Merck). The specimens were visualized under phase-contrast illumination to reveal cell morphology as well as staining, and bright field optics were used to clearly identify positive cells. Cultures treated with nonimmune serum, cultures not treated with first antibody, or cells which were cultured in the absence of cortisol were used as negative controls for these experiments. Since it was verified that RALA255-10G cells cultured at 33°C without cortisol did not synthesize tyrosine aminotransferase, antisem directed against this enzyme was preincubated twice for 15 min at room temperature with such cultures prior to use. By adopting this protocol, the background staining was significantly reduced.

RESULTS

Tyrosine Aminotransferase Activity in RALA255-10G Cells Grown at Permissive and Nonpermissive Temperatures. It has been demonstrated (5) that SV40 tsA255 mutant virus-transformed adult hepatocytes grown at the permissive temperature (33°C, transformed phenotype) synthesized low levels of albu-
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Fig. 3. Autoradiographs of Northern blots of poly(A)* RNA from RALA255-10G cells grown at 33°C and 40°C. The culture conditions were identical to those described in the legend to Fig. 2. Poly(A)* RNAs were separated by electrophoresis on 1.2% agarose gels containing formaldehyde and transferred to Zetabind membranes as described in "Materials and Methods." RNA was hybridized to 32P-labeled tyrosine aminotransferase complementary DNA, pcTAT3 (20), and β-actin complementary DNA. The amount of RNA applied was 5 µg for tyrosine aminotransferase (left) and 1 µg for actin (right), respectively.

In the presence of cortisol at 40°C, synthesis of tyrosine aminotransferase was greatly increased, and the synthesis was further enhanced by addition of Bt2cAMP (Fig. 2). In the absence of cortisol at 40°C, synthesis was not detectable either with or without Bt2cAMP. Tyrosine aminotransferase synthesis was therefore induced when RALA255-10G cells exhibited a nontransformed phenotype. However, glucocorticoid was absolutely required for expression. Bt2cAMP could enhance the induction by glucocorticoid, but Bt2cAMP alone was not sufficient to induce tyrosine aminotransferase synthesis. Tyrosine aminotransferase synthesized by RALA255-10G cells comigrated with the enzyme of normal rat liver (data not shown) with an apparent molecular weight of 55,000 (Fig. 2).

Tyrosine Aminotransferase mRNA Levels in RALA255-10G Cells Grown at Permissive and Nonpermissive Temperatures. Poly(A)* RNAs isolated from cells grown at 33°C and 40°C in the absence or presence of cortisol or cortisol plus Bt2cAMP were electrophoresed and hybridized with a tyrosine aminotransferase complementary DNA fragment (20). Fig. 3 shows that tyrosine aminotransferase mRNA in this temperature-sensitive adult hepatocyte cell line comigrated with the mRNA from normal rat liver. mRNA levels were very low in cells grown at 33°C even in the presence of cortisol, but Bt2cAMP greatly increased its steady-state level. Tyrosine aminotransferase mRNA was substantially increased in cells grown at 40°C in the presence of cortisol and cortisol plus Bt2cAMP (Fig. 3). Removal of cortisol from the culture medium decreased the level of tyrosine aminotransferase mRNA at both 33°C (data not shown) and 40°C (Fig. 3). To ensure that the results obtained with tyrosine aminotransferase were specific, and not

Biosynthesis of Tyrosine Aminotransferase by RALA255-10G Cells Grown at Permissive and Nonpermissive Temperatures. Labeling experiments were performed to determine whether tyrosine aminotransferase synthesis was induced when cells regained a nontransformed phenotype at 40°C. At 33°C, enzyme synthesis was not detectable either in the absence (data not shown) or presence of cortisol (Fig. 2). However, substantial amounts of enzyme were synthesized in the presence of both cortisol and Bt2cAMP.

At the nonpermissive temperature (40°C), the temperature-sensitive hepatocytes regained their nontransformed phenotype, and the synthesis of these liver proteins was greatly enhanced. However, while synthesis of transferrin was induced by glucocorticoid, synthesis of albumin was totally dependent on glucocorticoid. Expression of tyrosine aminotransferase, an intracellular liver-specific enzyme, was examined in RALA255-10G cells. In the absence of cortisol, enzyme activity was not detected in cultures grown at either 33°C or 40°C. Enzyme activity could be reliably measured only in cultures maintained at 40°C for 2 days in the presence of either cortisol or cortisol plus Bt2cAMP (Fig. 1). The data suggest that temperature shift-up induced the enzyme by Day 2 when cortisol or a combination of cortisol and Bt2cAMP was present. On subsequent days of culture, enzyme activity was below 40 units, which is the limit of sensitivity of the assay.

An experiment designed to test the effect of maintaining fetal hepatocyte cultures at 40°C versus 33°C on tyrosine aminotransferase activity showed that activity was reduced by 25, 35, and 83% after 1, 2, and 3 days of growth at 40°C, respectively. Therefore, the temperature-sensitive expression of tyrosine aminotransferase enzyme activity could not be conclusively demonstrated in RALA255-10G beyond 2 days of culture at 40°C.

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due to different amounts of mRNA applied to the Northern gels, duplicate samples of poly(A)+ RNA extracted from cultures maintained at 33°C and 40°C in the presence of cortisol and cortisol plus Bt2cAMP were probed with a β-actin complementary DNA. The results shown in Fig. 3 indicate that the quantity of actin mRNA in each sample was approximately the same.

Immunocytochemical Staining for Tyrosine Aminotransferase in RALA255-10G Cells Grown at Permissive and Nonpermissive Temperatures. Immunocytochemistry was performed on these cultures in order to define changes at the level of the individual cell. Phase-contrast photomicrographs of cultures maintained in the presence of cortisol or cortisol plus Bt2cAMP at 33°C or 40°C are shown in Figs. 4 and 5, respectively. In cultures maintained at 33°C in the presence of cortisol (Fig. 4b) or Bt2cAMP (data not shown), positive cells were very rarely seen. When present, such cells had a more dense cytoplasm, and staining by the antibody directed against the enzyme was usually relatively weak. Such cells represented less than 0.01% of the population. When Bt2cAMP was present together with cortisol, the relative number of positive cells increased to about 0.5%; moreover, they stained more intensely (Fig. 4d). Areas comprising cords of such cells displaying more granulated cytoplasm could be seen. However, not all cells stained positively for tyrosine aminotransferase. It is possible that such cells represented a population which escapes the virus-induced block in expression of differentiated properties. It is such cells which stained positively for albumin and transferrin, but their numbers were much greater than tyrosine aminotransferase-positive cells in cultures maintained at 33°C (about 0.1%).

In cultures which had been shifted to 40°C, there were many more cells which were granular in appearance and that were generally also larger (Fig. 5). The number of such positive cells was substantially increased when compared with cultures maintained at 33°C. Cell counts revealed that this increase was of the order of 100-fold (Fig. 4b versus 5b), so that between 1 and 2% of cells were positive in cultures exposed to cortisol alone.

Fig. 4. Immunocytochemical localization of tyrosine aminotransferase in RALA255-10G cells grown at 33°C. Two sets of cultures were grown at 33°C in medium containing 1 μM cortisol. The first set of cultures was maintained in cortisol for the duration of the experiment. The second set of cultures was treated with cortisol plus Bt2cAMP (0.5 mM) 4 days after cells reached confluence (Day 4). After an additional 2 days of growth in cortisol or cortisol plus Bt2cAMP, the cultures were fixed and stained for tyrosine aminotransferase as described in “Materials and Methods.” a, cortisol, nonimmune serum; b, cortisol, immune serum; c, cortisol + Bt2cAMP, nonimmune serum; d, cortisol + Bt2cAMP, immune serum.
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Fig. 5. Immunocytochemical localization of tyrosine aminotransferase in RALA255-10G cells grown at 40°C. Two sets of cultures were grown at 33°C initially in medium containing 1 μM cortisol. They were shifted from 33 to 40°C after 4 days of growth at 33°C (Day 0). Subsequent treatment was as described in the legend to Fig. 4. a, cortisol, nonimmune serum; b, cortisol, immune serum; c, cortisol + Bt2CAMP, nonimmune serum; d, cortisol + Bt2CAMP, immune serum.

Bt2CAMP alone did not induce an increase in the number of positive cells in 40°C cultures (data not shown). However, when Bt2CAMP was present together with cortisol, the total number of cells which were clearly positive was increased, and they now represented nearly 10% of the population. At the same time, the intensity of staining of the positive cells was enhanced compared with cultures exposed to cortisol alone (Fig. 5d). This suggests a greater enzyme synthetic activity per cell.

Discussion

Cells of various lineages such as myoblasts (1), chondroblasts (2), melanoblasts, (3), trophoblasts (4), and hepatocytes (5, 6) have been transformed by temperature-sensitive mutants of RNA or DNA tumor viruses. As a result, expression of the differentiated properties of the respective cells is suppressed. However, the expression of tissue-specific genes is restored by culturing these cells at the appropriate temperature. These cell systems are therefore suitable models to study the relationship between differentiation and cancer. The SV40 tsA255 mutant virus-transformed rat adult hepatocyte line RALA255-10G (5), temperature sensitive for maintenance of the differentiated liver phenotype, represents such a model. RALA255-10G cells synthesize low levels of albumin and transferrin when cultured at the permissive temperature of 33°C (transformed phenotype). At the nonpermissive temperature of 40°C (nontransformed phenotype), the production of both plasma proteins is substantially increased. Under both conditions, glucocorticoid hormone is necessary for the retention of differentiated functions.

In the present study, we demonstrated that expression of the tyrosine aminotransferase gene in RALA255-10G cells is also temperature sensitive. In cells grown at 33°C, enzyme activity, synthesis, and mRNA accumulation were low. However, expression of the tyrosine aminotransferase gene was greatly increased when these cells were shifted to 40°C. Like albumin synthesis in RALA255-10G cells, expression of the tyrosine aminotransferase gene was dependent upon the presence of glucocorticoid hormone. In the absence of glucocorticoid hormone, tyrosine aminotransferase levels were very low or undetectable. Bt2CAMP alone was not sufficient to induce expression...
of this enzyme; however, it greatly enhanced the induction by glucocorticoid. In all instances, the increase in mRNA levels paralleled the increase in synthesis. We are presently trying to determine whether such increase is due to a higher transcription rate of the tyrosine aminotransferase gene, the stabilization of the mRNA, or a combination of both mechanisms. We were unable to detect a complete correlation between increased activity and the upward temperature shifts, because of the thermal inactivation of tyrosine aminotransferase activity at 40°C. Although RALA255-10G cells are temperature sensitive in expression of the tyrosine aminotransferase gene, cells grown at 33°C produced considerable amounts of this enzyme and enzyme mRNA in the presence of cortisol plus Bt2cAMP. Thus, reversal of transformation by the temperature shift is a quantitative rather than a qualitative effect.

Tyrosine aminotransferase has been extensively studied both in vivo and in vitro because it is liver specific and is regulated by a variety of hormones including glucocorticoids, glucagon, adrenalin, and insulin. Expression of tyrosine aminotransferase in vivo is induced by administration of glucocorticoid (10, 11, 22) or cAMP (12). Since it is not possible to perform in vivo studies in the total absence of glucocorticoid, it remains unclear whether the cAMP response is dependent upon glucocorticoid. In some hepatoma cells, low levels of enzyme can be induced by glucocorticoid (23), and this level can be further enhanced by the addition of cAMP (24). However, there are some hepatoma cell lines in which cAMP is able to induce tyrosine aminotransferase in the absence of glucocorticoid (22). In primary cultures, tyrosine aminotransferase expression appears to be totally dependent on glucocorticoid (25), and an enhancement of expression by cAMP is observed (25). It can be concluded from this study that the capacity to synthesize the liver-specific enzyme tyrosine aminotransferase is retained by RALA255-10G cells. Furthermore, regulation of this enzyme by glucocorticoid and Bt2cAMP in these cells resembles that found in vivo and in primary cultures.

Immunocytochemical studies demonstrated that the increase in tyrosine aminotransferase gene expression at 40°C in the presence of glucocorticoid, or glucocorticoid plus Bt2cAMP, resulted from an increase in both the number of cells capable of producing this enzyme and the amount of tyrosine aminotransferase synthesized per cell. There are two mechanisms by which more tyrosine aminotransferase-positive cells can arise: (a) cells unable to produce this enzyme now become competent; (b) cells which were producing such low levels of enzyme as to be undetectable by the procedure used are now producing sufficient amounts of enzyme as to facilitate detection. The present experiments do not distinguish between these possibilities. To the best of our knowledge, this is the first reported study of immunolocalization of tyrosine aminotransferase in individual cells, and it shows that cultures of RALA255-10G cells are heterogeneous with respect to the synthesis of this enzyme.

The results of this study indicate that a virus-transformed cell line such as RALA255-10G is useful as a model for studying the reverse relationship between expression of the transformed and differentiated states. The mechanisms which lead to transformation appear to suppress the expression of genes which are characteristic of the cell lineage. However, the cell retains the capacity to reexpress these genes when the transformed phenotype is reversed. With respect to tyrosine aminotransferase activity when reexpression is initiated, its regulation by hormones closely resembles that observed in vivo and in primary cultures of hepatocytes.

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