Transformation-induced Alterations in the Regulation of Tyrosine Aminotransferase Expression in Fetal Rat Hepatocytes: Changes in Hormone Inducibility and the DNase-hypersensitive Site

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ABSTRACT

Regulation of expression of tyrosine aminotransferase (TAT) is examined in two cell lines (FRL) obtained by chemical transformation of cultured fetal hepatocytes derived from 19-day rat fetuses (FL19). Steroid induction of TAT is unaffected by transformation, while the response to cyclic AMP is attenuated. Consequently a synergistic response elicited by the simultaneous exposure of normal fetal hepatocytes to the inducers is almost abolished in FRL cells. FL19 and FRL are similar with respect to the negative effect of insulin on steroid induction, which is a response restricted to prenatal liver. Detailed examination of chromatin reveals that the attenuated effect of cyclic AMP is consistent with the lack of the DNase I-hypersensitive site located at about the cyclic AMP response element of the TAT promoter. From the studies, we conclude that transformation results in the modification of some aspects of TAT regulation, while others have been retained, and reflects the fetal pattern which is observed in normal embryonic hepatocytes.

INTRODUCTION

Our understanding of development has been greatly facilitated by the use of cell lines that retain the phenotype of different stages of various cell lineages. The usefulness of cell lines as representative models is most clearly seen in hematopoiesis, where many of the cell types arising from the bone marrow stem cell, including cells of the erythroid (1) and lymphoid (2) lineages, are now represented by cell lines. To obtain a cell line, a cell representing a particular lineage can be immortalized and usually transformed, and a determined program of phenotypic alterations, which will normally accompany cellular differentiation, may be interrupted as a consequence. Cell lines may therefore be representatives of the differentiated state of cells from which they are derived. However, it is possible that the process of transformation per se will alter the pattern of gene expression of the resulting transformed cell. Therefore, the cell line may be an imperfect representative of the original precursor. Changes in the regulation of particular genes which accompany the deviation of a given cell type from a determined path to an immortalized cell line could provide useful information on regulatory mechanisms which are responsible for or are a consequence of the transformed state.

Previous studies of liver development indicate that fetal rat liver hepatocytes are determined from at least day 13 to undergo a program of sequential acquisition of liver-specific characteristics (3–5) as the cells mature. These studies have been performed with primary cultures and provide a set of markers by which hepatocytes can be classified with respect to their stage of development. One such informative marker is TAT. The TAT gene has been extensively investigated because its expression is liver cell specific as well as developmentally and hormonally regulated. Although largely absent in fetal liver, TAT mRNA and enzyme activity appear very rapidly after birth (6, 7). It is of interest to note that its appearance can be prematurely induced by surgical delivery of rat fetuses (8). TAT is hormonally regulated, with considerable increases in its level observed as a consequence of glucocorticoid (9, 10) or glucagon administration, which act via cyclic AMP (10–13). TAT can be induced by in utero administration of glucagon or cyclic AMP. This induction is significantly enhanced by coadministration of cyclic AMP and glucocorticoids, but glucocorticoids alone have a negligible effect (14). There are interesting differences between adult and fetal hepatocytes in terms of the hormonal regulation of TAT. For instance, simultaneous exposure of fetal hepatocytes in culture to glucocorticoids and cyclic AMP leads to a synergistic induction of TAT expression, whereas in adult hepatocytes the effect of the two inducers is only additive (15, 16). While insulin has been reported to induce TAT in adult rats in vivo (17), in perfused liver (18), and in culture (19), in fetal hepatocyte cultures it inhibits the induction by dexamethasone (20).

Recent attempts to raise cell lines retaining characteristics of the rat fetal hepatocyte include transformation of primary cultures using simian virus 40 (21) and exposure to the hepatocarcinogen 3’-methyl-4-dimethyl-aminoazobenzene (22). By using this hepatocarcinogen, our laboratory has produced the FL19 cell lines (termed “FRL cells”) which possess a variety of liver markers, including the plasma proteins transferrin and α1-acid glycoprotein and liver-specific isoenzymes of pyruvate kinase and aldolase as well as tyrosine aminotransferase (23).

This study was undertaken to more accurately assess the differentiated status of FRL cells, particularly with respect to TAT expression. We examine the status of the TAT gene and its expression in the FRL cell lines as a criterion for determining the extent to which FRL cells have retained or deviated from a fetal hepatocyte phenotype. Recently, several mechanisms involved in the transcriptional control of the TAT gene have been elucidated. These include the characterization of DH sites which correlate with cell-specific expression (24) and defining 5’ regulatory sequences which are critical in controlling its level of activity. This study involves a detailed analysis of TAT expression in FRL cells. The levels of enzyme, mRNA, and transcription are determined and compared with levels in the normal counterpart, i.e., 19-day gestation fetal hepatocytes (FL19). We have, by DH site analysis of the TAT gene, established which cis elements of the TAT gene are active or inactive in FRL cells. This detailed analysis allows us to identify alterations from the determined path of fetal hepatocyte differentiation induced by immortalization and thus to begin a molecular characterization of this perturbation.

MATERIALS AND METHODS

Cell Culture Conditions and Additions to the Medium. Primary cultures of fetal rat hepatocytes derived from 19-day gestation rats were prepared and maintained as described previously (4). FRL cells were produced by exposing primary cultures of fetal hepatocytes derived from 19-day gestation rats (henceforth described as FL 19 cells) to 3’-methyl-4-dimethyl-aminoazobenzene as previously reported (22). Transformed colonies of hepatocytes were selected on the basis of morphology and subcultured using stainless steel cloning rings. The cells used in this study are the first two lines obtained which were shown to retain TAT expression even after 20 passages, and are termed FRL1 and FRL2, respectively. All cells were established and maintained in medium comprising equal volumes of Dulbecco’s modified Eagle’s medium.
and Hams’ F12 supplemented with 10% fetal calf serum, dexamethasone (10^-7 M), and insulin (10^-8 M) in a humidified incubator at 37°C in 5% CO2 in air. The medium was replaced the following day after subculture and every 2 days thereafter. One day before the cells were harvested for assay, cultures were washed twice with Hanks’ balanced salt solution and then placed in standard medium without hormones followed by the appropriate additions for test samples. Test samples received insulin (10^-8 M) or glucocorticoid (10^-7 M) and/or cyclic AMP analogues (10^-3 M).

**TAT Assay.** Cells were harvested in Hanks’ balanced salt solution by scraping the dishes with a Teflon policeman. Preparation of cytosol and TAT assay was carried out as described previously (4). The procedure involves ion exchange chromatography of each cytosolic extract so as to exclude aspartate aminotransferase, which is present at very high levels in fetal liver extracts. At these levels, the amount of tyrosine transaminated by aspartate aminotransferase significantly increases the quantity of [H]tyrosine which is transaminated (4). The protein content of cytosolic extracts was determined using the dye-binding method of Bradford (25), using bovine serum albumin as a standard.

**Immunocytochemistry.** Cultured cells were washed three times with PBS and fixed for 6 min at 4°C with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodilyle buffer (pH 7.45). This was followed by three washes with PBS and storage in 0.1 M cacodilyle buffer (pH 7.45) at 4°C. The indirect immunoperoxidase detection of TAT was performed according to the method of Clement et al. (26). The fixed cells were washed three times in PBS and incubated for 15 min with 0.1 M lysine in PBS-saponin (0.2%), followed by a 1-h incubation in 10% fetal bovine serum in PBS-saponin. Then circular areas of cells (about 5 mm in diameter) were made by the removal of surrounding cells using cotton buds. This method enabled a single culture to be tested with a variety of antibodies. Each circle of cells was then reacted with a 1:200 or 1:400 dilution of TAT antibody or nonimmune rabbit IgG for 1 h. Subsequently, the cells were washed with PBS-saponin three times, and a 1:200 dilution of the second antibody (peroxidase-coupled goat IgG directed against rabbit IgG) was added and incubated for 1 h. The washing procedure was repeated followed by a final wash in 0.05 M Tris (pH 7.5). A solution of 0.05% diaminobenzidine and 0.01% H2O2 in 0.05 M Tris (pH 7.5) was added and allowed to react for 20 min. The cells were then washed with PBS. In all experiments, cells exposed to only the second antibody or to the IgG fraction of nonimmune serum were incorporated as negative controls.

**Northern Blots.** Total RNA was prepared by the method of Cathala et al. (27) from cells treated with dexamethasone (10^-7 M) and/or 8-(4-chlorophenylthio)-cAMP (1 mM; Boehringer Mannheim) as specified for 5 h. Ten µg of each sample were electrophoresed through formaldehyde-agarose gels, blotted, and hybridized as previously described (24).

**Run-on Transcription.** Nuclei were prepared from cells treated with dexamethasone (10^-7 M) and 8-(4-chlorophenylthio)-cAMP as specified for 2 h. The preparation of nuclei, incorporation of [α-32P]UTP, purification of nascent RNA, and hybridization to immobilized plasmid DNAs were performed as previously described (10).

**Nuclease Hypersensitivity.** Nuclei were prepared from cells treated as for run-on transcription assays and subjected to DNase I digestions and indirect end-labeling analysis as described previously (28). Nuclei from primary cultures of fetal hepatocytes were treated identically, except that several strokes in an ice-cold, loose-fitting Dounce were performed after the hepatocytes had been in the Nonidet P-40-containing nuclear isolation buffer for 5 min. The nuclei were harvested by centrifugation 5 min after this step.

**RESULTS**

**Tyrosine Aminotransferase Enzyme Activity in FRL Cells.** Initially the effects of glucocorticoids, cyclic AMP, and insulin on TAT enzyme activity in FRL 1 and FRL 2 cell lines were examined (Fig. 1, A and B). These two cell lines were independent isolates, and we performed all experiments on both in order to ascertain whether they displayed the same or differing phenotypes of TAT expression. The FRL cell lines were derived from primary cultures of 19-day fetal hepatocytes (FL 19 hepatocytes); the inducible characteristics of TAT enzyme activity in such primary cultures are also shown (Fig. 1C). Both FRL 1 and FRL 2 cell lines show a very similar profile of induction. They are strongly inducible by glucocorticoids and weakly inducible by cyclic AMP (Fig. 1, A and B). Cotreatment with both glucocorticoids and cyclic AMP leads to a greater induction of TAT enzyme activity than is observed for the individual inducers. The effect of insulin on both cell lines is again very similar. Insulin has no significant effect on the uninduced or cyclic AMP induced levels; however, a small but reproducible decrease in the magnitude of glucocorticoid inducibility as well as the induction by both glucocorticoid and cyclic AMP are observable. In contrast, FL 19 hepatocytes show a slightly lower level of enzyme activity in the uninduced cultures and a similar magnitude of induction by glucocorticoids. Cyclic AMP is more effective, and the synergistic effect of the two inducers added together is more dramatic than observed with FRL lines (Fig. 1C). Thus the expression characteristics of TAT have been altered during...
immortalization of the FL19 hepatocytes in that the cells display an attenuated response to cyclic AMP (when it is added alone or in combination with dexamethasone). FRL cell lines and FL 19 hepatocytes both display a decreased magnitude of glucocorticoid inducibility in the presence of insulin. Insulin has no effect on cyclic AMP induction, and the decrease in synergism is most readily explained as a consequence of the decreased glucocorticoid component.

Immunocytochemical studies show that the changes in TAT activity reflect changes in the amount of protein in the FRL cells. A low level of staining is observed in control cultures (Fig. 2a); a modest increase is observed when cyclic AMP is present (Fig. 2b), and more intense staining is seen in cultures exposed to dexamethasone (Fig. 2c). The most intense staining is seen in cultures exposed to both inducers (Fig. 2d). In all cultures there appears to be heterogeneity in the staining pattern, since some cells show higher levels of enzyme than others. This is particularly noticeable in cultures exposed to dexamethasone, either alone or in combination with cyclic AMP (Fig. 2c and d). To ascertain whether this heterogeneity was an artefact, we stained other areas of the same culture dish for transferrin (Fig. 2e). Since a homogeneous pattern was observed we concluded that the heterogeneity of TAT staining truly reflects different levels of TAT protein in various cells. Equivalent cultures maintained in the presence of insulin were also stained for TAT, and a reduction in intensity of staining in cultures exposed to dexamethasone or dexamethasone and cyclic AMP was observed (data not shown). A coculture of FRL 2 and FL 19 maintained in medium supplemented with dexamethasone shows that the level of TAT staining reflects the enzyme levels obtained for the two cell types; i.e., there is more enzyme in the FRL cells, which appear as single cells, than in the primary cells, which form clusters (Fig. 2f). Thus we conclude that the levels of TAT enzyme activity observed in Fig. 1 are representative of changes in TAT protein levels.

Changes in TAT Enzyme Activity Are Mediated at the Transcriptional Level. To identify a molecular basis for the differences observed in Fig. 1 between FRL cells and FL 19 hepatocytes, we performed Northern blots and nuclear run-on transcription assays. Northern analysis reveals that regulation of TAT expression in these cells, in control cultures as well as those supplemented with inducers, is also evident at the level of the mRNA coding for TAT (Fig. 3). Overall, changes in mRNA level were consistent with those observed for enzyme activity. The inhibitory effect of insulin was also observed at the mRNA level. The induction by dexamethasone was reduced by about 50% when insulin is present, and as anticipated, cyclic AMP had only a marginal effect on mRNA levels in both FRL 1 and FRL 2.

Run-on transcription analyses were performed with nuclei prepared from cultures of FRL 1 and FRL 2 (Fig. 4). Here we observe that changes in TAT enzyme and mRNA levels in response to glucocorticoids, cyclic AMP, and insulin in FRL 1 and 2 cell lines are attributable to changes in the rate of TAT gene transcription. It is important that the nuclear run-on assay demonstrates that insulin decreases the induction of TAT gene transcription by glucocorticoids.

Differences in TAT Gene Chromatin between FRL Cells and Fetal Hepatocytes. The above analysis demonstrates that, in FRL cells, TAT is regulated primarily at the transcriptional level. We have shown previously that in primary cultures of fetal liver hepatocytes, TAT is also primarily regulated at the transcriptional level (29, 30). Much progress has been made recently in elucidating the transcriptional control mechanisms of the TAT gene (24, 31). These analyses were primarily based on the identification of the cis elements involved by DH site mapping of TAT gene chromatin. Fig. 5a presents a summary of DH sites observed in various hepatoma cell lines and adult liver (for a details see Nitsch et al., Ref. 24). In hepatoma cell lines and adult liver, the basal level of TAT gene expression is characterized by DH sites at the promoter and at an enhancer situated 10.5 kilobases upstream. In adult liver and in H4-II-E-C3 cell lines (24), an increased basal level of TAT gene transcription corresponds to an active enhancer and DH site at ∼3.6 kilobases upstream. Cyclic AMP stimulates TAT gene transcriptional activity, primarily through a cyclic AMP response element lying within the ∼3.6 DH site. Glucocorticoids stimulate TAT gene transcriptional activity by inducing the appearance of a DH site and active enhancer at ∼2.5 kilobases upstream (28).

We therefore determined the status of the TAT gene in FRL 1 and 2 cells by looking for the presence of these DH sites. To ascertain a mechanistic basis to explain the differences of TAT expression in FRL cells and FL 19 hepatocytes, we determined the status of TAT gene chromatin in FL 19 hepatocytes as well as in FTO cells. FTO cells are derived from the H4-II-E-C3 hepatoma cell line, and the pattern of TAT expression and DH sites have been previously shown to be more similar to adult hepatocytes than to the HTC hepatoma cell line (24) and are included in this study as a reference. Fig. 5 (b and c) compares DH sites observable in FRL cells, FL 19 hepatocytes, and FTO cells. Consistent with the presence of a basal level transcription observed in Fig. 4, a DH site at the promoter is present in all cases. It is weakest in uninduced cells, which show the lowest TAT activity (Fig. 5b, Lane FL 19 Un: Fig. 1). Glucocorticoids induce a DH site at ∼2.5 in all cases (∼2.5; Fig. 5b; Ref. 28). Insulin, which decreases the magnitude of TAT induction by glucocorticoids, does not appear to influence the formation or intensity of the glucocorticoid-dependent DH site (24). A DH site observable in FTO cells and adult liver is present at 1 kilobase upstream (∼1.0) but is absent in both FRL cell lines and FL 19 hepatocytes.

Fig. 5c shows DH sites observable further upstream of the TAT gene promoter. A site present in FL19 hepatocytes and FTO cells at ∼3.6 is absent in both FRL 1 and 2 cell lines. This site, which is also present in adult liver, marks an enhancer that increases basal, steady-state mRNA levels and contains a CRE (32). In FRL cells, the absence of this site corresponds to the absence of cyclic AMP inducibility. A site at ∼4.4 shows similarity to the site at ∼1.0, since it is present in FTO cells but not in FRL and FL 19 cells. DH sites at ∼5.4 and ∼11 are present in all cases. These sites are present whenever the TAT gene is active. The ∼10.5 site marks a liver-specific enhancer (24).

DISCUSSION

These experiments utilize fetal rat hepatocyte cell lines (FRL) produced by exposing fetal rat hepatocyte cultures to the hepatocarcinogen 3′-methyl-4-dimethylaminoazobenzene. These cells have been shown to synthesize the plasma proteins transferrin and α1-acid glycoprotein and to express liver-specific isoenzymes of pyruvate kinase and aldolase as well as tyrosine aminotransferase (23). This communication reports the results of a detailed study of TAT expression in FRL cells to determine changes with respect to TAT regulation, particularly with respect to the pattern of hormone inducibility after transformation.

Dexamethasone induces enzyme, mRNA, and transcription levels and a DH site at ∼2.5 (the GRE) in all cases. In both FRL cell lines and FL 19 hepatocytes, insulin suppresses this induction. In these respects the cell lines resemble the cells from which they are derived. The results also show that the levels of enzyme activity, protein, the abundance of mRNA, and transcription of the TAT gene correlate under the various induction conditions, and it is therefore reasonable to conclude that regulation of TAT is principally affected at the level of transcription in these cells. Hormonal regulation of TAT in fetal hepatocytes differs from that of adult cells in two important respects. First, the effect of glucocorticoid and cyclic AMP is additive in hepatocyte cultures established from adult rats (10), whereas in fetal hepatocyte cultures the agents together elicit a synergistic response to...
Fig. 2. Immunolocalization of TAT in FRL1 cells maintained in control medium (a) and in medium supplemented with cyclic AMP (b), dexamethasone (c), or dexamethasone plus cyclic AMP (d). FRL cells were also stained for transferrin (e). A mixed culture of FRL2 and FL19 stained for TAT shows strongly positive FRL2 cells (individual dark staining) and less intense staining of groups of FL19 cells (f).
which is considerably greater than the sum of the response obtained with each agent alone (29). Second, in adult hepatocytes, insulin has been reported to be an inducer of TAT in isolated hepatocytes (11) or to have a minimal effect on adult hepatocyte cultures (33), which contrasts with the situation in fetal hepatocytes where it significantly reduces the induction of TAT by dexamethasone (20). FRL cells do not show a synergistic response to glucocorticoid and cyclic AMP, unlike FL19 cells; however, they appear to respond to insulin-like fetal hepatocytes.

The attenuated response of FRL cell lines to cyclic AMP is supported by the finding that these cells lack a DH site at -3.6 kilobases, which marks an enhancer that is inducible by cAMP. It is also interesting to note that while cyclic AMP increases the level of enzyme by about 2-fold in FRL cells, it does not significantly increase either the level of mRNA or the rate of transcription of the gene. This suggests that the cyclic AMP effect in FRL cells is mediated at the posttranscriptional level. These studies also show that FRL 1 and FRL 2, which are different cell lines, are identical with respect to regulation of the TAT gene, but in certain respects they are unlike FL19 hepatocytes.

The expression of tyrosine aminotransferase has not been consistently observed in transformed hepatoma cell lines. Many of these have not retained the capacity to produce TAT, while others, such as the Morris hepatoma line HTC (34) and the Reuber hepatoma line H-4-II-E-C3 (35), are good producers of the enzyme and display a basal level of transcription and inducibility by glucocorticoids like primary cultures of adult hepatocytes. HTC cells differ from Reuber-derived lines and adult hepatocytes in that they show a lower basal level of TAT mRNA, are poorly inducible by cyclic AMP, and lack the -3.6 enhancer and DH site (24). The FRL cell lines display the same characteristics. This suggests that, with respect to TAT expression, the immortalization of HTC, FRL1, and FRL2 cell lines resulted in the loss of similar characteristic(s). HTC cells were derived from an ascites tumor derived from a solid hepatoma induced by feeding male
Buffalo rats a diet containing N,N'-2,7-fluorenylene-bis-2,2,2-trifuoroacetamide (33). H-4-II-E-C3 cells are derived from a Reuber H-35 hepatoma which was induced by feeding N2 fluorenyldiacetamide to a male A × C rat (36). It is possible that different patterns of TAT regulation result from transformation of different precursor cells.

Nuclease-hypersensitive analysis of the 5' upstream region of the TAT gene has revealed several features of interest. In general, the pattern is similar in FRL and FL19 cells, which is in turn similar to that found for FTO cells, which are derived from the H-4-II-E-C3 hepatoma cell. In a previous study (24), the pattern in FTO cells was shown to be similar to that in adult liver, and important hypersensitive sites were located at −10.5, −3.6, −2.5, and −1.0 kilobases and at the promoter. The prominent sites located at −10.5 and −2.5 kilobases (the GRE) and the promoter are present in all three cell types. Intensity of the ORE and the promoter correlates well with induction with dexamethasone and the level of expression, respectively. A difference was observed at the −3.6-kilobase site. This is absent in FRL cells and present in FTO cells and FL19 cells, and this finding suggests that although its presence correlates extremely well with expression (24), it is not an absolute requirement for expression. HTC cells derived from a Morris hepatoma also lack the −3.6-kilobase site. More detailed analysis of sequences at the −3.6-kilobase site by Boshart et al. (32) indicates that a region comprising about 80 base pairs confers cell-specific expression to a construct of this fragment coupled to the CAT reporter gene. The CRE also resides within this region (24), and this may explain the relative ineffectiveness of cyclic AMP in inducing TAT in FRL cells. The other nuclease-hypersensitive site which shows variation is located at around −1 kilobase. This is weak or absent in FRL cells and FL19 cells but shows up strongly in chromatin obtained from FTO cells.

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It was of interest to ascertain whether the inhibition of the dexamethasone induction of TAT by insulin is reflected in the intensity of the hypersensitive site which corresponds to the GRE. The results presented in this study reveal that in both FRL as well as FL 19 cells in which this phenomenon has been described (20, 29), there is no detectable alteration in the GRE site. This observation suggests that
insulin is not attenuating the glucocorticoid response by acting directly at the chromatin level, although this and a previous study (29) show that it acts to modify the level of transcription.

In summary, these studies show that transformed fetal hepatocytes retain some characteristics which are peculiar to fetal hepatocytes and distinct from adult cells, such as the inhibition of dexamethasone induction by insulin. However, transformation has produced changes in the chromatin structure, resulting in a loss of the CRE hypersensitive site, which accounts for the attenuated induction of TAT by cAMP in the transformed cells.

ACKNOWLEDGMENTS

The substantial contribution by A. F. Stewart and E. Schmid from the Institute for Cell and Tumor Biology at the German Cancer Research Center in Heidelberg is gratefully acknowledged. The transcription and nuclease-hypersensitive assays were performed with their help and instruction during Dr. Yeoh's tenure as a visiting scientist in the laboratory of Prof. G. Schutz.

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