

# Hepatocyte Differentiation In Vitro: Initiation of Tyrosine Aminotransferase Expression in Cultured Fetal Rat Hepatocytes

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**Abstract.** A fetal rat hepatocyte culture system has been used to study the molecular mechanisms of tyrosine aminotransferase (TAT) gene expression during development. It has previously been shown that TAT activity can be detected in 19-d, but not 15-d, gestation hepatocytes on the first day of culture (Yeoh, G. C. T., F. A. Bennett, and I. T. Oliver. 1979. *Biochem. J.* 180:153-160). In this study enzyme activity, synthesis, and mRNA levels were determined in hepatocytes isolated from 13-, 15-, and 19-d gestation rats maintained in culture for 1, 2, or 3 d and exposed to dexamethasone. TAT expression is barely detectable in 13-d gestation hepatocytes even after 3 d in culture. Hepatocytes isolated from 15-d gestation fetuses have undetectable levels of enzyme activity and synthesis on the first day of culture; both can be assayed by days 2

and 3. TAT mRNA levels in these hepatocytes, measured by hybridization with a specific cDNA, increase substantially during culture. TAT activity, synthesis, and mRNA are evident on the first and subsequent days of culture in 19-d gestation hepatocytes. Transcription measurements in isolated nuclei indicate that the increase in TAT mRNA in 15- and 19-d gestation hepatocytes is associated with an increase in transcription of the gene. Immunocytochemical studies demonstrated that the increase in TAT expression correlated with an increase in the proportion of hepatocytes expressing the enzyme, rather than a simultaneous increase in all hepatocytes. These results support the proposal that a subpopulation of 15-d fetal hepatocytes undergo differentiation in culture with respect to TAT.

**T**YROSINE aminotransferase (TAT)<sup>1</sup> is one of a group of rat liver enzymes that first appears in the postnatal liver (12, 33). TAT expression is virtually absent in the fetus; enzyme activity (33) and mRNA (25, 26) accumulate shortly after birth when transcription of the gene is activated (26). Although inactive in the fetus, the TAT gene is capable of expression before birth. TAT activity can be precociously induced by in utero injection of glucagon or dibutyryl cAMP (13, 25, 27), premature delivery of the fetus (19), as well as in fetal liver explants (27, 28, 35) or monolayer cultures (36). It is suggested that fetal hepatocytes undergo a differentiative event before birth rendering the TAT gene potentially expressible (36). The absence of expression during gestation is probably due to an inhibitory component(s) within the uterine environment (3, 18, 25).

To study TAT expression in fetal liver, a hepatocyte culture system, which has been shown to be suitable for enzyme production in competent hepatocytes, was used (36). Dexamethasone, capable of inducing TAT in fetal liver maintained in vitro, was added to cultures to facilitate detection of the enzyme. Using this system, it has previously been demon-

strated that hepatocytes derived from fetuses as young as 16-d gestation possess TAT activity if placed in culture for 1 d (36). 15-d gestation hepatocytes and younger do not have detectable levels of enzyme activity under similar conditions of culture. It was concluded in this study that hepatocytes at ~16-d gestation undergo a differentiation event in vivo resulting in the TAT gene becoming capable of expression. 15-d fetal hepatocytes appear to be able to undergo this event when maintained in culture. Although enzyme activity is not detectable on the first day of culture, it is acquired by day 3. In this study, TAT gene expression in 15-d fetal hepatocytes maintained in culture for 1, 2, or 3 d has been investigated with respect to enzyme synthesis, mRNA levels, and gene transcription. In addition, immunocytochemical studies were undertaken to investigate events occurring at the cellular level. Similar biochemical and immunocytochemical measurements in 13- and 19-d gestation hepatocytes, developmental ages both preceding and subsequent to the putative differentiation event, have been included for comparison. Hepatocytes at all stages of gestation have been maintained in a constant environment with identical periods of exposure to dexamethasone at all time points. In this way, it is concluded that the observed difference in TAT expression between hepatocytes is an inherent property of the cell.

1. *Abbreviations used in this paper:* GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; TAT, tyrosine aminotransferase.

## Materials and Methods

### Animals

Rats of the Wistar strain of *Rattus norvegicus* which have a 22-d gestation period were used. Gestational age was determined by vaginal smear to detect spermatozoa and is accurate to  $\pm 8$  h.

### Isolation and Culture of Fetal Hepatocytes

Fetal hepatocytes used for culture were obtained from livers of 13-, 15-, or 19-d gestation rats. Livers from 19-d gestation rats were incubated with collagenase (0.5 mg/ml balanced salts solution [15], 0.17 U/mg, Boehringer Mannheim, GmbH, FRG) for 15 min at 37°C and prepared for culture as previously described (36). The isolation procedure for 15-d gestation livers involved a 5-min incubation with collagenase before being disaggregated by passage through a Pasteur pipette (8). Cells isolated from the equivalent of seven livers were resuspended in 10 ml of medium. Livers from 13-d gestation fetuses were removed by microscopic dissection techniques, treated with collagenase (0.5 mg/ml balanced salts solution [15], 80 livers/ml), prepared in the same manner as 15-d gestation hepatocytes and cells derived from 70 livers resuspended in 10 ml of medium, and dispensed into collagen-coated culture dishes. All fetal hepatocytes were maintained in MEM supplemented with 10% FCS and antibiotics (36). Dexamethasone, prepared as a 1,000-fold stock solution in propylene glycol, was added to hepatocyte cultures 18 h before harvesting or fixation to give a final concentration of  $10^{-7}$  M. Hepatocytes not exposed to the glucocorticoid analogue received an equivalent volume of vehicle for the same time period.

### Enzyme Activity Assay

To accurately measure TAT activity in fetal hepatocytes, it was necessary to first remove aspartate aminotransferase, an enzyme capable of tyrosine deamination, from cell extracts. Aspartate aminotransferase was separated chromatographically from TAT using CM-Sephadex C-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) ion-exchange mini columns (36). A radiochemical method described previously (36) was then used to measure TAT activity. 1 U catalyzed the formation of 1  $\mu$ mol of *p*-hydroxyphenylpyruvate/h at 37°C. The DNA content of the samples was measured by the fluorometric method of Hinegardner (17).

### TAT Synthesis and Immunoprecipitation

Hepatocyte cultures were incubated at 37°C in methionine-free medium for 150 min before harvesting to diminish intracellular levels of methionine. After 60 min of incubation, L-[<sup>35</sup>S]methionine (1,200 Ci/mmol; New England Nuclear, Boston, MA) was added to cultures. Subsequently, cells were harvested, extractable polypeptides immunoprecipitated with specific antisera according to the method of Gross et al. (14) incorporating the modifications of Fletcher et al. (8). Immunocomplexes were analyzed by SDS-PAGE in slab gels (22) and detected by fluorography as described by Bonner and Laskey (2). Bands corresponding to TAT, identified by fluorography, were cut from the gel, solubilized, and the radioactivity was determined by liquid scintillation counting in a  $\beta$  counter (LS 3800; Beckman Instruments, Inc., Palo Alto, CA).

### Preparation of Total RNA and Hybridization

Total RNA was isolated from tissue or cell cultures by lithium chloride/urea extraction (24). For Northern analysis, 20  $\mu$ g of RNA was electrophoresed in a 1.2% agarose gel containing 2.2 M formaldehyde (23) and electroblotted onto GeneScreen (New England Nuclear, Boston, MA) using a Transblot cell (Bio-Rad Laboratories, Richmond, CA). For dot blots, total RNA was denatured in 2.2 M formaldehyde, 1 M NaCl, 0.1 M tri-sodium citrate, pH 7.0, and blotted onto Hybond nylon transfer membrane (Amersham, Bucks, UK). TAT or glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was detected by hybridization with pTAT-3 cDNA (30) or pRGAPDH-13 cDNA (9) clones, respectively. These were labeled with deoxycytidine 5'-[ $\alpha$ -<sup>32</sup>P] triphosphate (3,000 Ci/mmol, Amersham) using a nick translation kit (Bethesda Research Laboratories, Gaithersburg, MD). Hybridization conditions included 50% formamide, 5 $\times$  SSC, 0.1% SDS, 5 $\times$  Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA), and 250  $\mu$ g/ml denatured salmon sperm DNA at 46°C for 36 h after an overnight prehybridization. Membranes were washed (10 min/wash) with vigorous agitation at room temperature (unless specified) with (a) 2 $\times$  SSC,

0.1% SDS; (b) 0.5 $\times$  SSC, 1% SDS at 65°C; and (c) 0.5 $\times$  SSC, 0.1% SDS; and then exposed to X-ray film (RXO-H; Fuji, Hamamatsu, Sydney, Australia) at -70°C with an intensifying screen.

### "Run-on" Transcription in Isolated Nuclei and Hybridization

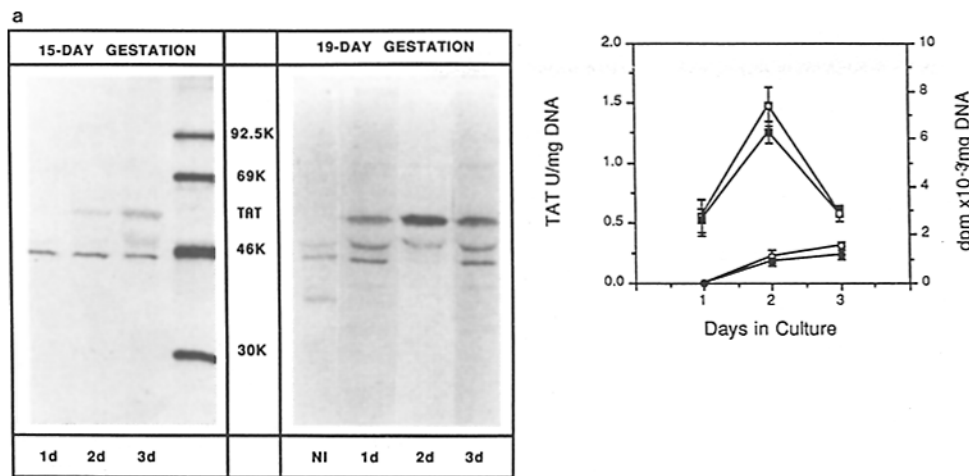
Nuclei were isolated from fetal hepatocyte cultures by the method of Becker et al. (1), suspended in 60% glycerol, 4 mM MnCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM DTT, 20 mM Tris, pH 7.4, and stored at -20°C. Transcription in isolated nuclei, isolation of <sup>32</sup>P-labeled RNA, and hybridization to nitrocellulose filters were performed as described previously (5) with some modifications. Briefly, isolated nuclei ( $\sim 10^7$ ) were incubated in a reaction mixture consisting of 50 mM Hepes (pH 8.0), 150 mM NH<sub>4</sub>Cl, 1 mg/ml BSA, 1 mM MnCl<sub>2</sub>, 12.5% glycerol, 0.1 mg/ml heparin, 3.5 mM MgCl<sub>2</sub>, 1  $\mu$ l (60 U) RNasin (Boehringer Mannheim GmbH), 0.25 mM DTT, 0.5 mM ATP, GTP, CTP, and UTP, 100  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P] UTP (3,000 Ci/mmol, Amersham) for 20 min at 25°C. The mixture was adjusted to final concentrations of 5 mM Tris (pH 7.5), 5 mM MgCl<sub>2</sub>, and 5 mM CaCl<sub>2</sub>; and 1U RQ1 DNase (Promega Biotec, Madison, WI) was added and the mixture incubated for 5 min at 37°C. After treatment with proteinase K (150  $\mu$ g/ml) at 37°C for 30 min, RNA was isolated by phenol-chloroform extraction followed by TCA precipitation and ethanol precipitation in the presence of 0.05 mg/ml transfer RNA. After treating again with RQ1 DNase as above, RNA was isolated by phenol-chloroform extraction, collected by ethanol precipitation as above, and hybridized at 37°C for 72 h in 55% formamide, 4 $\times$  SSC, 0.1 M sodium phosphate (pH 6.8), 1 $\times$  Denhardt's solution, 0.1% SDS, 100  $\mu$ g/ml salmon sperm DNA, and 10% dextran sulphate to 0.25 pmol of each of the following nitrocellulose-bound DNA: (a) TAT genomic subclones TAT EH 0.95, TAT EH 2.45, and TAT EE 1.05 (34); (b) albumin genomic subclones "B," "C," and "D" (29); (c) GAPDH cDNA pRGAPDH-13 (9); (d) phosphoenolpyruvate cDNA pPCK10 (38); (e) cDNA for mouse  $\alpha$ 1-antitrypsin (6); and (f) pUC8. Filters were washed at 65°C three times in 1 $\times$  SSC, 0.1% SDS (15 min/wash) and then twice in 0.1 $\times$  SSC, 0.1% SDS (45 min/wash) and associated radiolabel was visualized by fluorography and assessed by optical densitometry using a video densitometer-model 620; Bio-Rad Laboratories. Densitometric signals were converted to relative transcription rates by subtracting the background signal (pUC8 signal) and correcting for the fraction of primary transcripts hybridizable to the recombinant genomic DNA. Corrected values for albumin or TAT were normalized relative to the corrected pRGAPDH-13 signal for the respective filter. To enable an indirect comparison of transcription rates of TAT and albumin, the corrected and normalized values were divided by the respective gene length giving the relative proportion of transcripts produced per unit time from that gene, to the number of GAPDH transcripts produced per unit time.

### Immunocytochemistry

Fetal hepatocyte cultures were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde and stained for TAT as described (4), with the following modifications: rabbit IgG specific for rat TAT or transferrin was diluted 1:200 or 1:500 and peroxidase-coupled goat-Fab anti-rabbit IgG (Institut Pasteur, Marnes, France) diluted 1:200. Peroxidase activity was revealed by reaction with diaminobenzidine (0.5% in 0.05 M Tris, pH 7.5) in the presence of 0.3% H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature. Parallel incubations with IgG isolated from nonimmunized rabbits were included as negative controls. The number of hepatocytes expressing TAT were counted and expressed as a percentage of the total number of hepatocytes. As  $\sim 500$  cells were counted per culture, and this relied upon identification of hepatocytes by light microscopy, the results can only be regarded as semiquantitative and approximate.

## Results

It has previously been demonstrated that hepatocytes isolated from fetuses 16-d and older have low levels of TAT activity when maintained in culture; this activity is enhanced by exposure to dexamethasone (36). To facilitate detection of TAT, all hepatocytes in this study, unless otherwise noted, were exposed to maximally inducing levels of dexamethasone 18 h before harvesting or fixation. Control cultures, which did not receive dexamethasone, were included in all experiments



**Figure 1.** TAT activity and synthesis levels in 15 and 19 d fetal hepatocytes in culture. (a) Electrophoretic analysis of <sup>35</sup>S-labeled TAT. After one, two, or three d in culture, hepatocytes derived from 15-d or 19-d gestation fetuses were incubated in the presence of L-[<sup>35</sup>S]methionine for 90 min, anti-TAT-precipitable polypeptides immunoprecipitated with specific antisera, analyzed by SDS-PAGE, and detected by fluorography as described in Materials and Methods. A parallel incubation with nonimmune serum (NI) was included. The band corresponding to TAT, which is reported to have a sub-unit molecular mass of 51

kD (11), was identified by comparison with known <sup>14</sup>C-methylated protein standards: phosphorylase B (92.5 kD), BSA (69 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD). (b) Hepatocytes derived from 15-d (○) and 19-d (□) gestation rats were maintained in culture for 1, 2, or 3 d and levels of TAT synthesis (●, ■) and activity (○, □) measured. TAT activity is expressed as TAT U/mg DNA; synthesis is presented as dpm × 10<sup>-3</sup>/mg DNA. Data represents the mean ± standard error of six determinations.

reported in this study, but the level of activity, mRNA, and staining by immunocytochemistry were always very low or undetectable.

### TAT Synthesis and Activity Levels

Labeling experiments were performed to determine if TAT was synthesized in 15-d fetal hepatocytes on the first day of culture when enzyme activity is not detectable. As shown in the fluorograph (Fig. 1 a), [<sup>35</sup>S]-labeled anti-TAT-precipitable polypeptides were not observed in these hepatocytes on the first day of culture. However, the radiolabeled product could be detected by days 2 and 3 and on each day of culture in 19-d gestation hepatocytes. Synthesis and activity levels in 15- and 19-d fetal hepatocytes maintained in culture for 1, 2, or 3 d are presented in Fig. 1 b. TAT synthesis levels correlated with enzyme activity measurements for both 15- and 19-d fetal hepatocyte cultures.

### Steady-state TAT mRNA Levels

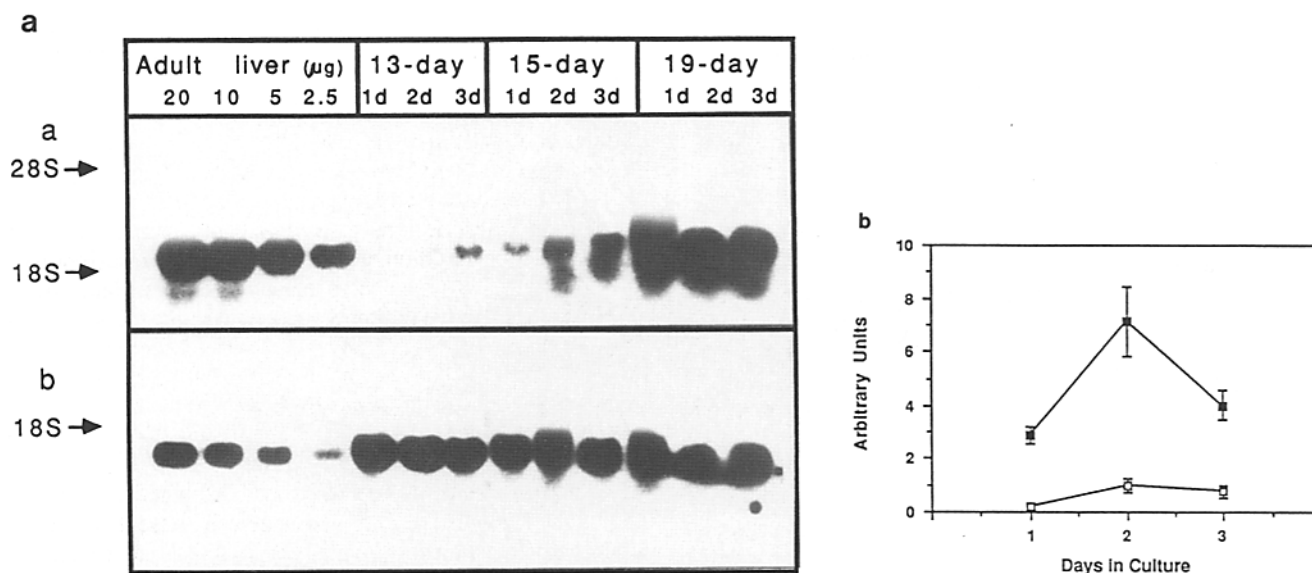
To determine steady-state levels of TAT mRNA, total RNA was isolated from fetal hepatocyte cultures, electrophoresed on a denaturing gel, electroblotted, and hybridized with a specific TAT cDNA clone (Fig. 2 a). Blots were exposed for several days to detect low levels of TAT mRNA. A single species of mRNA (2.4 kb) was revealed that appeared to be similar in size to TAT mRNA isolated from adult liver. GAPDH served as an internal control to ensure that similar amounts of total RNA from fetal hepatocyte samples had been analyzed. GAPDH mRNA levels did not differ appreciably between fetal samples (Fig. 2 a). Hepatocytes isolated from 13-d gestation rats did not have detectable levels of TAT mRNA on the first day of culture; very low levels could be observed by d 3. 15-d gestation hepatocytes had barely detectable levels of TAT mRNA on the first day of culture; however, these levels increased substantially when these hepatocytes were maintained in culture for 2 or 3 d. In contrast to 13- and 15-d gestation hepatocytes, TAT mRNA was present in 19-d gestation hepatocytes at appreciable levels on the first

and subsequent days of culture. By the second day of culture, the accumulation of TAT mRNA in 19-d gestation hepatocytes was estimated to be ~20% that of induced adult levels in vivo. TAT mRNA levels in 15- and 19-d gestation hepatocyte cultures were analyzed by dot blot hybridization and results from a representative experiment are presented in Fig. 2 b. Changes in TAT mRNA over the 3 d in culture were consistent with variations in TAT synthesis and activity during this time (Fig. 1 b).

### Transcription Measurements in Isolated Nuclei

To determine whether the increase in steady-state levels of TAT mRNA in cultured fetal hepatocytes was due to altered stability of the mRNA or a change in the rate of transcription of the gene, TAT transcription was analyzed by a run-on transcription assay. Hepatocytes from 15- or 19-d gestation hepatocytes were maintained in culture with or without exposure to dexamethasone. Nuclei were isolated and nascent RNA chains elongated in vitro in the presence of <sup>32</sup>P-UTP. Labeled RNA was hybridized to excess single-copy DNA from various genes that had been immobilized on nitrocellulose filters. A representative slot blot from one such experiment is illustrated in Fig. 3. Results, quantitated by densitometry, are presented in Table I. The relative rates of transcription of the TAT and albumin genes have been calculated relative to transcription of the constitutive gene GAPDH.

Marginal levels of TAT mRNA detectable in control cultures of 19-d gestation hepatocytes (data not shown) were reflected by low levels of transcription in these cells on the first and third day of culture. When 19-d gestation hepatocytes were exposed to dexamethasone, TAT transcription was markedly enhanced on the first as well as third day of culture. TAT transcription was not definitively detected in control cultures of 15-d gestation hepatocytes on the first day of culture. Even after exposure to dexamethasone, hybridization to TAT DNA was not reproducibly greater than nonspecific hybridization to the vector DNA in these cells. However, when 15-d gestation hepatocytes were maintained in culture for 3 d

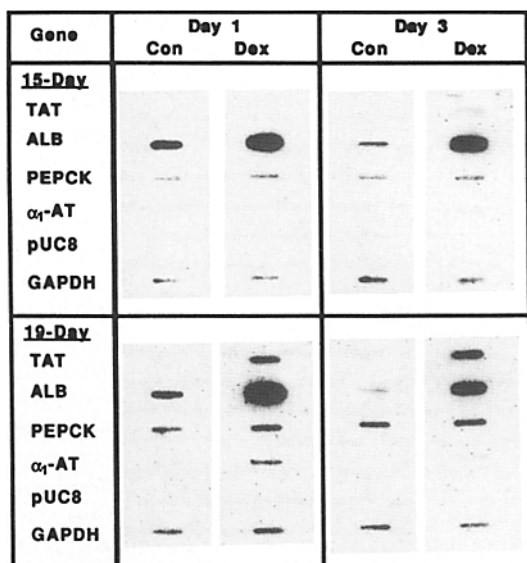


**Figure 2.** TAT mRNA levels in fetal hepatocytes in culture. (a) Total RNA (20  $\mu\text{g}/\text{lane}$ ) was isolated from 13-, 15-, or 19-d gestation hepatocytes on the first, second, or third day of culture, electrophoresed in a 1.2% agarose gel containing formaldehyde, electroblotted onto GeneScreen, and hybridized to  $^{32}\text{P}$ -labeled pTAT-3 cDNA or pRGAPDH-13 cDNA as described in Materials and Methods. Adult liver RNA, prepared in the same manner as fetal samples, was isolated from rats that had been injected intraperitoneally with dexamethasone (10 mg/100 g body weight) 5 h before. Ribosomal RNA bands of 18 S and 28 S were used as standards. (b) Relative quantitation of TAT mRNA in 15-d ( $\square$ ) or 19-d ( $\blacksquare$ ) gestation hepatocytes maintained in culture. Total RNA was isolated from fetal hepatocytes on the first, second, and third day of culture, and analyzed by dot blot hybridization as described in Materials and Methods. TAT mRNA levels were quantitated by densitometry and values, the mean  $\pm$  standard error of at least three samples, are indicated in arbitrary units. Data was normalized relative to GAPDH and mRNA levels.

and exposed to dexamethasone, TAT transcription was always detected. The differences in hybridization signal intensity between nuclear transcripts from 15- and 19-d gestation hepatocytes after 1 and 3 d of culture corresponded to differ-

ences seen in TAT mRNA levels at these times. These results indicate that TAT expression in cultured fetal hepatocytes appears to be controlled primarily at the level of transcription and is not accounted for simply by a change in the stability of TAT mRNA.

Albumin transcription was evident in control cultures of 15- and 19-d gestation hepatocytes on each day of culture, and was enhanced by exposure to dexamethasone. It is important to note that unlike TAT gene transcription, the rela-



**Figure 3.** Transcriptional analysis in nuclei isolated from 15- and 19-d fetal hepatocytes in culture. Hepatocytes isolated from 15- or 19-d gestation fetuses were maintained in culture for 1 d (Day 1) or 3 d (Day 3) with (Dex) or without (Con) exposure to dexamethasone as described in Materials and Methods. Nuclei were isolated, and nascent RNA chains were elongated in vitro and hybridized to immobilized DNA from the following genes: TAT, albumin (ALB), phosphoenolpyruvate carboxykinase (PEPCK),  $\alpha_1$ -antitrypsin ( $\alpha_1$ -AT), GAPDH, and pUC8.

**Table I.** TAT and Albumin Transcription in Isolated Nuclei Prepared from Fetal Hepatocytes in Culture

Sample	Vector signal	Hybridization signal*			Relative rate of transcription	
		TAT	GAPDH	Albumin	TAT	Albumin
<b>15-d gestation hepatocytes</b>						
d 1 Con	0.06	0.01	0.89	2.26	0.01	0.47
d 1 Dex	0.03	0.09	0.66	7.42	0.04	2.10
d 3 Con	0.02	0.02	0.85	.81	0.01	0.18
d 3 Dex	0.05	0.25	0.62	6.30	0.12	1.90
<b>19-d gestation hepatocytes</b>						
d 1 Con	0.05	0.10	0.62	1.74	0.04	0.53
d 1 Dex	0.15	1.06	0.75	9.07	0.40	2.25
d 3 Con	0.06	0.20	0.95	0.36	0.06	0.07
d 3 Dex	0.06	1.67	0.92	3.95	0.52	0.80

All numerical values refer to optical density units.

\* Data has been corrected for nonspecific hybridization by subtraction of vector signal.

† TAT and albumin densitometric signals were corrected for the fraction of primary gene transcripts hybridizable to the genomic DNA, normalized relative to GAPDH, and converted to relative rates of transcription as described in Materials and Methods.

tive rate of albumin transcription was enhanced in the presence of dexamethasone on the first day of culture in 15-d fetal hepatocytes. These results imply that the changes in TAT transcription are specific.

### ***Immunocytochemical Localization of Tyrosine Aminotransferase***

TAT localization in 13-, 15-, and 19-d gestation hepatocytes maintained in culture for 1, 2, or 3 d and exposed to dexamethasone, was investigated using a peroxidase-labeled second antibody directed against rabbit IgG specific for rat TAT. The specificity of staining for TAT was demonstrated at both the electron (data not shown) and light microscope level. Brown reaction product, indicating the presence of TAT protein, was localized within the cytoplasm of hepatocytes, never the nucleus or mitochondria. Nonparenchymal cells, present between islands of hepatocytes, did not stain for either TAT or transferrin (Fig. 4 *e-h*). In all experiments, negligible staining was observed when IgG from nonimmunized rabbits was used in place of the primary antibody.

13-d gestation hepatocytes did not appear to have any TAT-expressing cells on the first day of culture (data not shown). A few TAT-positive cells ( $\sim 1-2\%$ ) could be identified when these hepatocytes were maintained in culture for 3 d (data not shown). When 15-d gestation hepatocytes were maintained in culture for 1 d, the occasional TAT-positive cell ( $\sim 1-2\%$ , indicated by arrows) could be observed (Fig. 4, *a* and *b*). This finding is in accordance with very low levels of TAT mRNA detectable in these cells at this stage of development. By day 2, the proportion of positive cells increased markedly, and small clusters and cords of these cells could be found apparently randomly distributed among the cell population (Fig. 4, *c* and *d*). At this time, these hepatocytes represented only a minor portion of the total population ( $\sim 8-12\%$ ). Conversely, immunocytochemical staining for transferrin in 15-d fetal hepatocytes maintained in culture for 2 d indicated that almost all hepatocytes expressed transferrin (Fig. 4, *e* and *f*). Therefore cells expressing transferrin, but not TAT, were also considered to be hepatocytes. TAT-expressing cells were found in 19-d gestation hepatocytes maintained in culture for one (Fig. 4, *g* and *h*) or more days (data not shown), and appeared to represent a greater proportion of the hepatocyte population ( $\sim 15-18\%$ ) than observed in 2-d-old cultures derived from 15-d gestation liver (Fig. 4, *c* and *d*). However, at no time in culture did all hepatocytes, either 15- or 19-d gestation, express the enzyme; an apparently heterogeneous labeling pattern was always observed.

### ***Discussion***

The fetal hepatocyte culture system provides a useful model to study the molecular mechanisms of TAT gene expression during development. Although the TAT gene is potentially expressible in the fetus, expression is suppressed in the in utero environment. High circulating levels of insulin, known to be present in late gestation fetuses, have been implicated in suppression of TAT in utero (3, 18). By placing fetal hepatocytes in culture, thereby relieving restrictions imposed in vivo, it is possible to determine when the gene first becomes expressible during development. Experiments, based on enzyme activity measurements in fetal hepatocyte cultures (36), suggest that the gene can only be expressed from 16-d

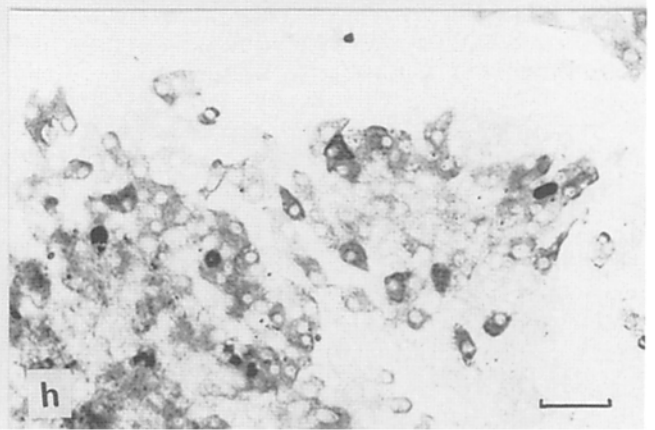
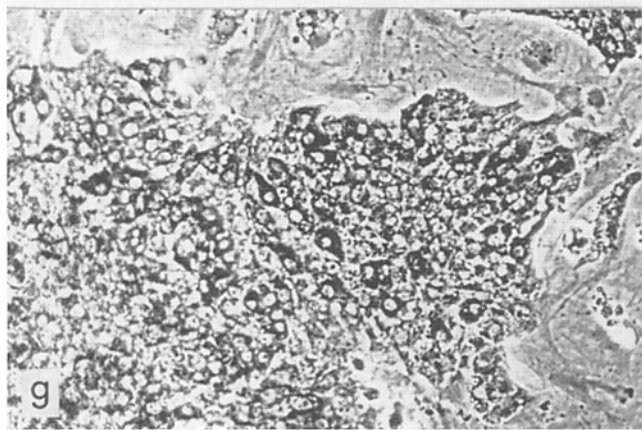
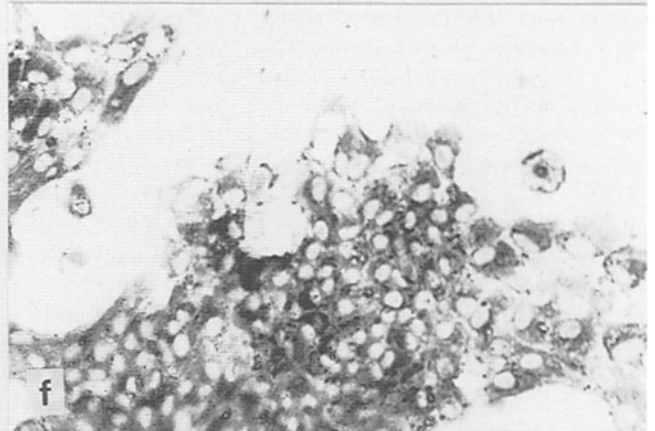
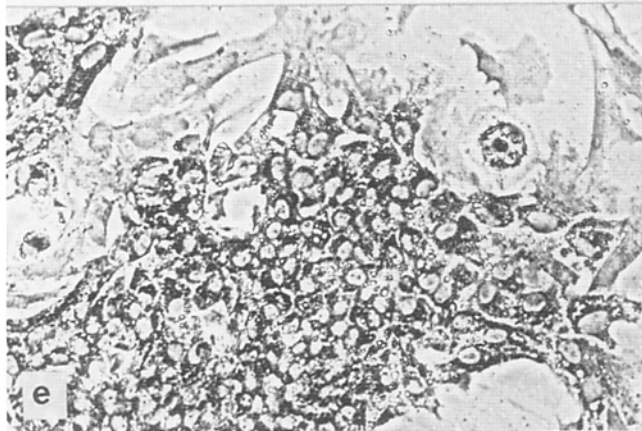
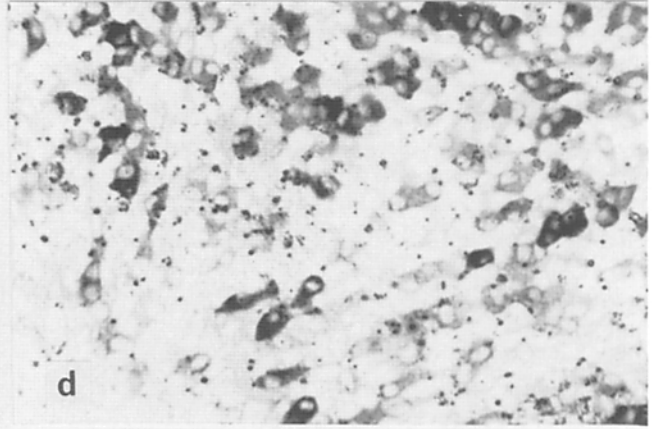
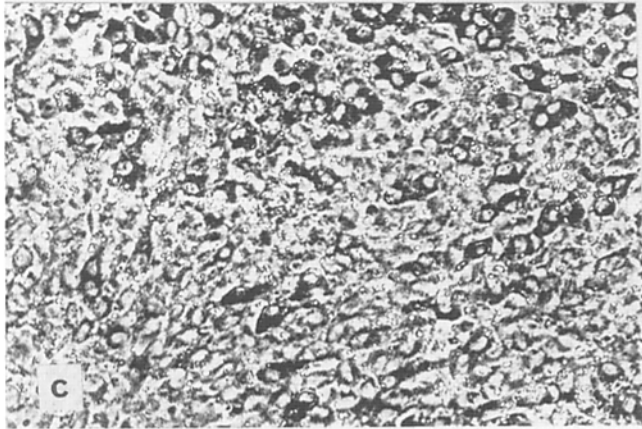
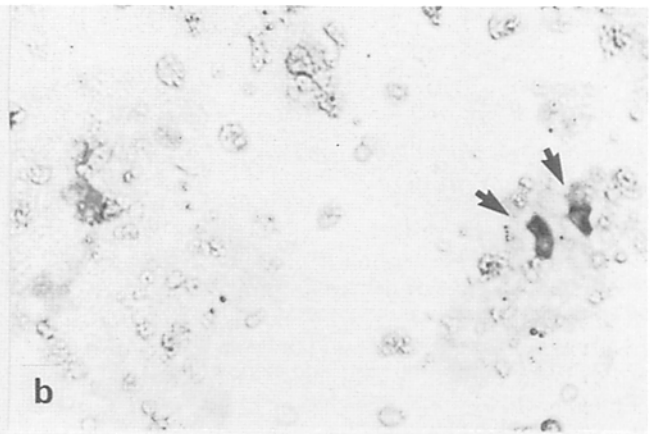
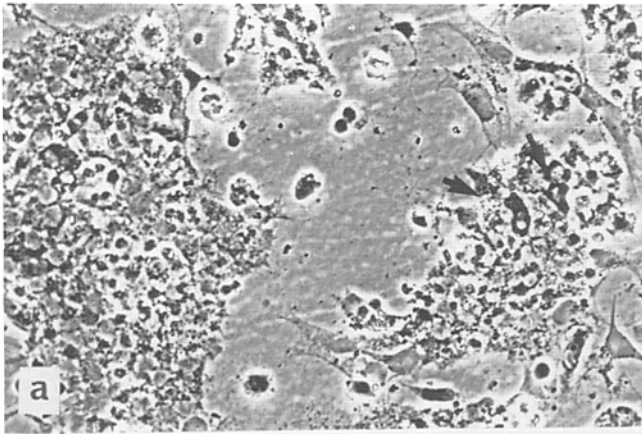
gestation onward. However, such studies do not preclude the possibility that the gene is, in fact, transcribed at an earlier stage of development. For example, the initial appearance of TAT activity may involve activation of an inactive precursor synthesized by immature hepatocytes.

This study was undertaken to investigate TAT expression at the level of enzyme activity, protein synthesis, and mRNA, as well as transcription, to establish the critical event(s) responsible for initiation of TAT expression. Transcription of the albumin gene was also assessed in nuclei isolated from different stages of development to serve as a control. Albumin transcription was evident in control cultures derived from 15- and 19-d gestation rats on both days 1 and 3. The relative rate of transcription was enhanced in the presence of dexamethasone.

Results from this study demonstrate that 15-d fetal hepatocytes acquire the capacity to express TAT when maintained in culture. Enzyme activity and synthesis, absent on day 1, could be assayed by days 2 and 3. TAT mRNA and transcription were barely, if at all, detectable on the first day of culture but increased substantially when these hepatocytes were maintained in culture for 2 and 3 d. Unlike 15-d gestation hepatocytes, TAT activity and synthesis were evident on the first and subsequent days of culture in hepatocytes isolated from 19-d gestation fetuses. This correlated with the presence of TAT mRNA on each day of culture in these hepatocytes, although levels were considerably lower than TAT mRNA determinations in induced adult liver. The increase in TAT mRNA in 15- and 19-d gestation hepatocytes maintained in culture was proportional to changes in transcription of the gene indicating that TAT expression in fetal hepatocytes maintained in vitro is controlled primarily at the level of transcription, as has been demonstrated for both intact and primary cultures of adult liver (16, 32) and the activation of TAT expression after birth (26). In view of these results, it is proposed that 15-d gestation hepatocytes undergo a differentiative event in culture such that the TAT gene becomes capable of transcription. Hepatocytes isolated from 19-d gestation fetuses appear to have already differentiated in vivo and are capable of TAT transcription when placed in culture.

Unlike their more mature counterparts, hepatocytes isolated from 13-d gestation fetuses did not have appreciable amounts of TAT mRNA, even after 3 d in culture. It is important to stress that in these experiments, hepatocytes derived from 13-, 15-, and 19-d gestation fetuses were maintained in a constant environment with identical periods of exposure to dexamethasone at all time points. The ability of the more mature hepatocytes to express TAT would appear to be due to inherent differences between the cells, not the exposure to dexamethasone. It is unlikely that the absence of TAT expression in immature hepatocytes reflects an inability of these hepatocytes to respond to glucocorticoids. This is supported by the finding that albumin transcription, unlike TAT transcription, is enhanced by exposure to dexamethasone in 15-d gestation hepatocytes on the first day of culture, suggesting that the machinery required for the glucocorticoid response is active.

Studies of radiation-induced mutations in mice have established that the deletion of a region at and around the albino (*c*) locus in chromosome No. 7 affects the hormone-inducible expression of TAT as well as several other liver



genes (10). Newborn mice homozygous for the deletion have a reduced level of TAT mRNA that is refractive to induction by either glucocorticoids or cAMP (31). It has been postulated that factor(s) encoded in the deleted region may be essential in conferring competence on the TAT structural gene to respond to these stimuli (7). The failure of glucocorticoids to induce TAT in fetal liver was interpreted as a lack of competence of the TAT gene in prenatal hepatocytes to respond to the hormone. However, results from this study indicate that the glucocorticoid analogue dexamethasone is capable of inducing TAT in hepatocytes as early as 15-d gestation when maintained in culture for 2 d. Furthermore, TAT activity is enhanced by both glucocorticoids and cAMP in organ cultures of 16-d gestation liver (28). On this basis, if the *trans*-acting factor(s) confers competence on the TAT gene to respond to hormones, it would appear to be present in fetal liver from at least 16- or 17-d gestation. It remains to be elucidated whether this *trans*-acting factor(s) is involved in the initiation of TAT expression in 15-d gestation hepatocytes maintained in culture.

The above biochemical measurements provided information about the cell population en masse. An immunocytochemical study was undertaken to investigate TAT localization at the cellular level. This study demonstrated that the increase in TAT expression, measured by biochemical means, was reflected by an increase in the proportion of hepatocytes expressing the enzyme, rather than a simultaneous increase in TAT expression in all hepatocytes. An increase in the amount of TAT synthesized per cell may also contribute to the overall increase in expression. Very few cells expressing TAT could be detected when 15-d gestation hepatocytes were maintained in culture for 1 d, consistent with low levels of gene activity detected at this time. By day 2, the proportion of hepatocytes synthesizing the enzyme had increased. It is likely that these cells represented a population of hepatocytes that differentiated in culture and in this respect became capable of expressing TAT. It has already been demonstrated that 19-d gestation hepatocytes are capable of TAT expression when placed in culture. Numerous TAT expressing cells were evident when 19-d gestation hepatocytes were maintained in culture for 1 d, these presumably have undergone differentiation *in vivo*.

Even after 3 d in culture, all hepatocytes derived from 19-d gestation fetuses did not express the enzyme (data not shown); a heterogeneous distribution pattern was observed. However it has been demonstrated that almost all hepatocytes are capable of expressing both albumin and transferrin from as early as 12-d gestation (21, 37). These findings lend support to the proposal that the developing liver is comprised of subpopulations of hepatocytes, or compartments of cells, which can be characterized by differential expression of liver proteins such as TAT. Cells enter a compartment by undergoing a differentiative event thereby acquiring the ability to ex-

press a new protein or proteins. In this way, hepatocytes are thought to transit from one compartment to the next during development such that a lineage of hepatocytes can be demonstrated (36). Myogenic and chondrogenic lineages have been characterized whereby cells pass through consecutive compartments within their respective lineages resulting ultimately in terminally differentiated muscle cells or chondrocytes (20). In the case of TAT, hepatocytes appear to be recruited into the TAT compartment from ~16-d gestation and this recruitment appears to occur in an asynchronous manner.

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**Figure 4.** Localization of TAT and transferrin in fetal hepatocytes in culture. When hepatocytes isolated from 15-d gestation fetuses were maintained in culture for 1 d (a and b), a few isolated hepatocytes (indicated by arrows) staining for TAT could be identified. By d 2 (c and d), the proportion of these cells increased such that a subpopulation of hepatocytes expressing the enzyme could be identified. However, when these 2-d-old cultures were stained for the presence of transferrin (e and f), almost all hepatocytes were positive. Hepatocytes isolated from 19-d gestation fetuses exhibited a large proportion of TAT-positive cells on the first day of culture (g and h). Micrographs show cells viewed under phase contrast microscopy (a, c, e, and g) and brightfield illumination (b, d, f, and h). Bar, 50  $\mu$ m.

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