

To the Graduate Council:

I am submitting herewith a dissertation by Robin Stephanie Rothrock entitled "Activation of Tyrosine Aminotransferase Expression in Hepatic Differentiation." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Francis T. Kenney
Francis T. Kenney, Major Professor

We have read this dissertation
and recommend its acceptance:

R L Lee

E. Volkin

Audrey Stevens

Wesley J. Wicks

Accepted for the council:

Cowminkel
The Graduate School

ACTIVATION OF TYROSINE AMINOTRANSFERASE
EXPRESSION IN HEPATIC
DIFFERENTIATION

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Robin Stephanie Rothrock

December 1983

ACKNOWLEDGMENTS

I thank my committee members, Audrey Stevens, Ken Volkin and Pete Wicks for their advice and constructive criticism regarding the direction of this project. In my lab, I'd especially like to thank Kai-Lin Lee for every μg of DNA I ever labeled, Alfred Johnson for every Laemmli he ever poured for me, and Ken Isham for invaluable hours of expert technical assistance. This work would not exist in its present form without the cooperation received from other groups. Jim Wesley did all the histology and histochemical analyses. Dave Allison and Marie Rorvik did the electron microscopy and the first class photomicrographs. Frank Kenney, my advisor, deserves special thanks. He provided the opportunity for me to do this work, told me to focus, did some of my enzyme assays, edited this dissertation, and instructed me that scientists never cry.

This research was supported by National Institutes of Health Research Grant HD 14173, the Office of Health and Environmental Research, United States Department of Energy, under contract with the Union Carbide Corporation, National Institutes of Health Training Grant GH 7438, and the Graduate Research Participation Program, Oak Ridge Associated Universities.

ABSTRACT

Elucidation of those molecular mechanisms regulating development and differentiation is a central question of contemporary biology and biochemistry. The developmental activation of the liver-specific enzyme tyrosine aminotransferase (L-tyrosine: 2-oxoglutarate aminotransferase, E.C. 2.6.1.5, TAT) has been qualitatively described in terms of the appearance of functional gene products and the role of effector molecules. The underlying developmental events to account for this activation have yet to be identified. It was the purpose of this investigation to analyze the activity of the aminotransferase gene during development. The project had two major areas of research. One involved the titration of hybridizable transcripts in perinatal rat liver using a cloned cDNA specific for messenger RNA^{TAT} (mRNA^{TAT}) sequences. The other used treatment of fetuses in utero with a potent inhibitor of DNA methylation, 5-azacytidine (5-azaC) to test whether the extent of specific DNA methylations could be implicated in the developmental activation of this gene. We present evidence here that supports our hypothesis that the rapid appearance of gene products in neonates is the consequence of an increased number of mRNA^{TAT} sequences. The data also suggest that the ability of those transcripts to be expressed is regulated post-transcriptionally, probably at the level of translation. Treatment of fetuses in utero with 5-azaC activated the expression of aminotransferase gene products in a pattern not unlike that seen in the course

of normal development. In addition, 5-azaC treatment induced changes in hepatic morphology and metabolism, apparently accelerating the developmental process. From these studies we conclude that the activation of tyrosine aminotransferase expression is probably the consequence of an increased rate of aminotransferase transcription. Superimposed on this is some form of post-transcriptional control which limits temporarily the capacity of the gene transcripts to be translated. Furthermore, we conclude that 5-azaC treatment in utero accelerates hepatic differentiation. The specific effect of this drug on TAT activation could be either on the structural gene itself or those hypothetical regulatory loci required for fully differentiated enzyme expression.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
II. ANALYSIS OF THE DEVELOPMENTAL ACTIVATION OF TYROSINE AMINOTRANSFERASE MESSENGER RNA SYNTHESIS.	10
III. ANALYSIS OF THE EFFECT OF 5-AZACYTIDINE ON HEPATIC DIFFERENTIATION	33
IV. SUMMARY.	56
LIST OF REFERENCES	60
VITA	68

LIST OF FIGURES

FIGURE	PAGE
1. Selection of Large mRNAs for Translation.	19
2. Electrophoretic Analysis of Immunoprecipitates of In Vitro Translation Products Directed by Size-Selected Poly(A) RNAs.	20
3. Hybridot Analysis of mRNA ^{TAT} Sequences in Perinatal Rat Liver	22
4. Integration Units Derived from Densitometric Scanning are Proportional to RNA Concentration.	23
5. Accumulation of Hybridizable Cytoplasmic mRNA ^{TAT} Sequences During Development.	25
6. Time Course of the Developmental Activation of Tyrosine Aminotransferase Expression.	26
7. Hybridizable Nuclear RNA Sequences Increase Immediately Prior to Birth.	28
8. Sucrose Gradient Analysis of mRNA ^{TAT} Sequence Distribution in Post-Mitochondrial Supernatants	30
9. Dose Response of Tyrosine Aminotransferase Activity	38
10. Time Course of Activation of Tyrosine Amino- transferase Expression.	41
11. Hybridot Analysis of mRNA ^{TAT} Accumulation Following 5-Azacytidine Treatment	43
12. Fetal Rat Liver Morphology.	45
13. Lipid and Glycogen Stores in Fetal Liver Visualized by Electron Microscopy	46
14. Sucrose Gradient Analysis of Fetal Poly(A) RNA.	48
15. Electrophoretic Analysis of Soluble Proteins from Fetal Liver.	50

CHAPTER I

INTRODUCTION

Studies in developmental biology have long focused on the central question of how a pluripotent fertilized egg can give rise to a complex organism of diverse cells and tissues. It is now generally accepted that the process of differentiation is the consequence of selective gene expression, i.e., activation of expression of particular subsets of genes and silencing of the remainder. Elucidation of the molecular mechanisms involved in the selective expression of genes, a major goal of contemporary biology, is thus considered to hold the promise of dispelling much of the mystery that has hitherto surrounded development and differentiation.

Knowledge of the structure and function of the eukaryotic genome has accumulated exponentially in recent years, owing in large part to the advent of recombinant DNA and other techniques of molecular biology that have enabled researchers to identify and characterize specific genes. These advances have led to the description of a variety of transcriptional and post-transcriptional control mechanisms that are operative in differentiating cells, but as of yet few of these can be integrated into generalizations of widespread validity. An apparent exception, of particular significance to the work described here, is the phenomenon of DNA methylation.

DNA Methylation: a Possible Molecular Mechanism
of Differentiation

In recent years much attention and speculation has been focused on the 5-methylcytosine (m^5C) residues of eukaryotic DNA and their potential function, especially concerning the regulation of differentiation, control of transcription and maintenance of chromosome structure. In any mammalian DNA only a small fraction (3.0 to 8.0%) of the total number of cytosine residues are present as m^5C , methylation occurring soon after DNA synthesis. Their distribution is not random, usually m^5C is 5' to a guanine residue (thus m^5CpG). Furthermore, the majority of these residues occur in repetitive DNA, not usually considered transcriptionally active, although a significant fraction of m^5C is associated with the nonrepetitive, structural gene DNA sequences. The distribution of m^5C residues within the DNA of a given organism is not constant over the course of development, the loss of specific m^5C residues has been associated with gene activity (1-7). An innovative approach by Naveh-Manny and Cedar (8) positively correlated active gene sequences, DNAase I sensitivity and hypomethylated DNA. Fractionation of sea urchin DNA with methylation sensitive/insensitive restriction enzyme isoschizomers defined two compartments on the basis of their DNA composition, methylated and nonmethylated. The transcriptionally active genes were found in the unmethylated DNA compartment of all samples analyzed (9).

Tissue specific patterns of methylation are correlated with the expression of tissue-specific genes. The classic analysis of the

human $\gamma\delta$ -globin locus by van der Ploeg and Flavell (10) and of the avian egg-white protein loci by Chambon and his associates (11,12) demonstrate that certain CpG dinucleotides, usually 5' to the structural gene, are demethylated in the DNA from the tissue in which the DNA is expressed. Housekeeping genes, such as adenine phosphoribosyltransferase and dihydrofolate reductase, those genes expressed in all cell types, have also been found with hypomethylated DNA regions (13). The DNA, again, is demethylated in the 5' flanking region of each gene, whereas the structural genes themselves are methylated at all sites tested.

Specific changes in the pattern of methylation have been correlated to the developmental expression of complex gene families. Expression of the α -globin gene cluster in terms of chromatin structure and methylation has been studied in developing chick embryos. During the switch from the primitive to the definitive lineage of erythroblasts, the U-gene (coding for the embryonic globin α -chain) becomes inactive. Inactivation is reflected by the generation of a more DNAase I-resistant structure, DNA methylation and cessation of transcriptional activity (14). The development of estrogen sensitivity by the major vitellogenin gene of avian liver is associated with the appearance of a specific demethylation event, approximately 600 base pairs 5' to the start site of transcription. However, transcription of the gene begins prior to the detection of the demethylation event in either embryonic or adult male liver (15,16). Demethylation is also thought to be one of the regulatory mechanisms involved in immunoglobulin heavy chain class-switching (17).

The appeal of specific DNA methylations as a means of regulating gene expression is quite persuasive--modification of DNA structure can be inherited, but is also reversible. Evidence for both maintenance methylase (18) and demethylase (19) activities in mammalian cells is accumulating.

Treatment with 5-azacytidine (5-azaC), a compound that is a potent inhibitor of DNA methylation but has several other biological effects (20), has been found to reactivate an inactive X-chromosome (21), induce differentiation (22-24), facilitate retroviral expression (25), modulate metallothionein I induction by heavy metals (26), and induce expression of previously expressed genes in vivo (27). These effects on specific gene expression and differentiation for the most part can be traced to 5-azaC induced hypomethylation of DNA. 5-Azacytidine is thought to effect demethylation by direct incorporation into the DNA (the 5-azaC residue cannot be modified) or by incorporation into the DNA and a subsequent irreversible inhibition of methyltransferase activity when the enzyme interacts with the modified base (28).

The majority of the evidence to date positively correlates hypomethylation of specific DNA sequences to the expression of certain genes. The consensus of opinion is that specific demethylation is a necessary but not sufficient condition for transcription. If demethylation is a necessary, but not sufficient condition for transcription, then it is implied that activation is the consequence of an ordered sequence of events at the molecular level prior to expression. Evidence for this is coming from chromatin studies of

genes, such as avian α -globin (14), and vitellogenin II (15), whose expression is developmentally regulated. Changes in chromatin structure may precede demethylation events, which may or may not precede the initiation of transcription.

Morphological and Molecular Differentiation: Perinatal

Rat Liver as a Model System

Many different aspects of mammalian liver development have been examined, including cellular composition, growth, enzymic/biochemical characteristics, and hormonal status. Integration of this information provides a multidimensional framework in which to analyze a particular aspect of hepatic differentiation.

Fetal rat liver functions first as a hematopoietic organ. However, after 14 days of gestation, the hematopoietic function decreases as hepatocytes increase in both size and number. Between 15 and 18 days of gestation, erythroid elements still predominate, contributing two-thirds of the total cell number and representing approximately one-half the total hepatic volume. At term, the situation is reversed, one-half the total number of cells are hepatocytes, and they fill approximately 88% of the total liver volume (29). The wet weight of the tissue increases approximately one and one-half times during the last day of gestation. After birth, hepatic wet weight decreases markedly for several days, largely the consequence of depleted glycogen stores. Hematopoietic elements are typically cleared by the fifth day following parturition and the liver begins another period of rapid growth between days 10 and 28 (30,31).

One aspect of differentiation is the acquisition of tissue specific enzymes. It is generally recognized that it is the development of a characteristic, quantitative pattern of enzymes that dictates any given tissue's physiological function and morphologic appearance. Two features of this developmental activity are significant. One is that the appearance of an enzyme is often not gradual, accumulation to adult levels occurring dramatically, within hours or days. The other is that the appearance of the liver enzymes so far studied tend to happen in clusters; in rat liver there are three critical periods, late fetal (16 to 22 days in utero), neonatal (one day after birth), and late suckling (21 to 28 days after birth) (32). In adult mouse liver, there are 10 to 12 different, tissue-specific, abundant messenger RNAs (mRNAs). Translation and RNA-DNA hybridization studies for five of those sequences show that not all members of this class are under common developmental regulation. Barth, et al. define at least three periods when liver-specific, abundant mRNAs appear: late fetal, neonatal and weaning (33). These data are in good agreement with those periods of development of enzyme activities characterized in rat liver.

Much of the work done in hepatic enzymic differentiation (34) has been directed towards a description of "developmental triggers" to account for the observed patterns of developmental activation. The emphasis has been on the induction of enzyme activity by hormonal or other physiological effector molecules. This approach was grounded in the knowledge of adult patterns of enzyme activity and induction.

In utero, the fetus is in a glucose rich environment, glucose freely crossing the placental barrier from the maternal circulation. Under these conditions, the molar ratio of insulin to glucagon in fetal blood is very high (10 to 1). Following parturition, the situation changes rapidly. The newborn animal must make the immediate transition from a continuously fed state to a typical cycle of feeding and fasting. This is usually associated with an initial, though transient, period of hypoglycemia, with a concomitant increase in glucagon levels and plummeting levels of insulin, reversing the previous ratio. Blood glucose levels are elevated by mobilizing glycogen stores and by de novo glucose synthesis from lactate, pyruvate, glycerol and amino acids (35). Another environmental change arises from the markedly different nature of the nutrients available in milk, once nursing begins. Milk is a low carbohydrate, high-fat, protein rich food. In rat milk, only 8.0% of the calories come from lactose, whereas 26.5% come from fat (36).

In developing rat liver, glucocorticoids are capable of effecting both inductive and regressive responses. Greengard considers two periods of extremely high steroid levels as critical to the further maturation of hepatic tissue: the accumulation of corticosteroids during days 18 and 19 of gestation, and then again, beginning about 10 days post-partum. The fetal adrenal gland begins to secrete glucocorticoids starting at about 16 days gestation, and the total level of glucocorticoids peaks at a level six-fold above that in the maternal circulation on day 19. The level then declines to that of the maternal circulation by the time of birth. In both fetal and neonatal

liver, glucocorticoids are thought to effect the involution of the hematopoietic elements (37,38).

Tyrosine Aminotransferase: a Model System for the Study
of Hepatic Differentiation

Tyrosine aminotransferase (E.C. 2.6.1.5, TAT) is often presented as a paradigm for the study of tissue-specific developmental activation. Synthesis of this enzyme in fully differentiated liver is modulated by glucocorticoids and is also sensitive to both insulin and glucagon, the latter acting through cAMP. The aminotransferase is synthesized at a very high rate under steady state conditions, but a similarly rapid rate of intracellular turnover maintains a low steady state level of activity. These unusual properties contribute to the interest surrounding this highly regulated enzyme of intermediary metabolism (39-43).

The enzyme is present at very low levels in fetal liver (~1 unit per mg protein) and enzyme synthesis is refractory to induction by glucocorticoids in utero (44). Insulin is similarly without effect in fetal liver, and treatment of fetuses late in gestation with cAMP has only small effects on enzyme activity (45,46). In the hours immediately after birth, enzyme activity increases rapidly, peaking several fold above adult steady state levels between 12 and 18 hours post-partum, and thereafter declining to adult levels (16 to 20 units per mg protein) between 24 and 36 hours (44). Glucocorticoid sensitivity is also acquired following release from the intrauterine environment. However, the development of adult levels of activity

occurs independently of glucocorticoid induction (44,47). Fetal hepatocytes, when placed in short-term organ culture, not only increase their steady state level of TAT specific activity, but also gradually acquire sensitivity to glucocorticoid induction (48). The acquisition of functional mRNA^{TAT} activity, as determined by in vitro translation and immunochemical titration, increases in parallel with that of enzyme activity (47).

Thus, the working hypothesis of TAT developmental activation has been as follows. Some mechanism of intrauterine repression exists, resulting in a low level of expression that is refractory to hormonal modulation; release from that environment permits the gene to be expressed. All the data imply that activation is probably the consequence of an increased rate of transcription. The molecular mechanisms underlying the activation event remain to be elucidated.

The principal objective of this research was to further define the sequence of events involved in the activation of TAT expression during liver differentiation. This approach has used a cloned cDNA to chemically titrate the TAT transcripts independently from their function. In the course of these experiments, the effect of the hypomethylating agent, 5-azaC, was examined. The results strongly implicate DNA methylation as a highly significant component of the activation of TAT expression and other aspects of hepatic differentiation.

CHAPTER II

ANALYSIS OF THE DEVELOPMENTAL ACTIVATION OF TYROSINE AMINOTRANSFERASE MESSENGER RNA SYNTHESIS

Introduction

Early in gestation the fetal rat liver is primarily a hemato-
poietic organ. However, as fetuses approach term, the hepatocyte
component increases in both size and number (29). Hepatocellular
development is accompanied by enzymic differentiation, the acquisition
of tissue-specific metabolic/biochemical functions in a regulated,
temporally ordered pattern (32). Tyrosine aminotransferase is one of
a number of liver-specific enzymes whose expression is markedly acti-
vated in neonatal rat liver.

Specific aminotransferase activity is very low in fetal liver; in
the hours immediately following birth, functional enzyme accumulates
rapidly, peaking at approximately 12 hours post-partum, then declining
to adult steady state levels by 24 to 36 hours (44). Release from the
uterine environment is also followed by an increase in the transla-
tional capacity of the mRNA^{TAT} , as detected by in vitro translation
and immunochemical titration (47); functional mRNA^{TAT} then accumu-
lates in concert with the neonatal increase of TAT activity. Under
typical assay conditions, the fetal level of mRNA^{TAT} translational
activity is not reliably detected. Thus, in order to demonstrate the
presence of this functional gene product in fetal liver, extraordinary
assay conditions were devised.

Development of a cloned cDNA specific for mRNA^{TAT} sequences has made possible the analysis of gene transcripts independent of function. This type of measurement permits a more direct assessment of TAT gene activity. Hybridization analyses were done throughout the perinatal period to quantitate transcripts in both cytoplasm and nuclei. These yielded the surprising result that cytoplasmic transcripts are present at adult levels well before the mRNA is functional either in the hepatocyte or in a heterologous translation system.

Preliminary experiments were done to probe the basis for the inactive mRNA^{TAT} sequences in newborn rat liver. There is evidence in the literature that some mRNA molecules are maintained in an inactive state in a nonpolysomal RNA-protein compartment in the cytoplasm (49,50). Testing this, the distribution of hybridizable mRNA^{TAT} sequences in post-mitochondrial supernatants was analyzed. These results do not explain the inactive mRNA^{TAT} transcripts.

Experimental Procedures

Animals

Virgin female Sprague-Dawley (strain CD/CR) rats (200 to 225 g from Charles River Breeding Laboratories) were placed with males overnight. Early the following afternoon, females were tested by vaginal smears for the presence of spermatozoa; those testing positive were considered to be in day one of gestation. Delivery is usually on day 22. Fetuses were taken for analysis early in the morning of the appropriate day. Precise age of newborns was established by direct observation of their birth.

Isolation of Cytoplasmic Poly(A) RNA

Livers from fetal or newborn rats were homogenized in three volumes 0.25 M sucrose plus polysome buffer (PB; 25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 5 mM MgCl₂). The homogenates were centrifuged at 300 x g for 10 minutes, then 12,000 x g for 10 minutes, and finally at 27,000 x g for 20 minutes, at 4°C. Supernatants were taken and heparin added to 0.5 mg/ml. Polysomes and RNA-protein complexes were precipitated by the addition of MgCl₂ to 100 mM and incubation at 4°C for 90 minutes, as described by Palmiter (51). The precipitate was collected by centrifugation at 27,000 x g for 15 minutes, suspended in 0.25 ml 0.25 M EDTA and solubilized in 1.0% sodium dodecyl sulfate (SDS). Poly(A) RNA was isolated by one passage through a one to two ml oligo(dT)-cellulose column (52). The RNA was precipitated by the addition of Na-acetate to 0.23 M and two and one-half volumes absolute ethanol (ETOH) and stored at -20°C.

Sucrose Gradient Fractionation of Poly(A) RNA

Five to 20% sucrose (w/v) gradients in 20 mM Na-acetate, pH 5.5, 5 mM EDTA, and 0.5% SDS were generated in 5.0 ml SW50.1 nitrocellulose tubes. The poly(A) RNA was heated for 3 minutes at 80°C in 0.2 ml sterile H₂O and layered onto the tops of the gradients. The gradients were centrifuged at 40,000 rpm for 210 minutes, in a SW50.1 rotor (Beckman) at 20°C. After centrifugation, the gradients were fractionated from top to bottom, 0.3 ml fractions collected, and the absorbance read at 254 nm. RNA from each fraction was precipitated by the addition of Na-acetate to 0.23 M and two and one-half volumes ETOH and stored at -20°C.

Cell-Free Protein Synthesis and Immunochemical Analysis

Poly(A) RNAs were assayed for mRNA^{TAT} activity by in vitro translation in a reticulocyte lysate system. The lysate was prepared as described by Pelham and Jackson (53) and treated with micrococcal nuclease to destroy endogenous mRNA activity. Prior to assay, each lysate was titrated to determine the optimal K⁺ and Mg⁺⁺ concentrations, reaction time, and concentration of added poly(A) RNA giving a linear response in the incorporation of [³H] leucine into protein. In a typical 100 μ l reaction, containing 50 μ Ci [³H] leucine (40-50 Ci/mmol, Amersham), 2.0 μ g poly(A) RNA was assayed. After incubation for 3 hours at 30°C, a small aliquot was removed for determination of total protein synthesis by the method of Mans and Novelli (54). The remainder of each sample was then used for specific immunoprecipitation with antibody prepared against purified aminotransferase, followed by analysis of the immunoprecipitates by SDS-polyacrylamide gel electrophoresis (Weber-Osborn gels, from 55) and quantitation as previously described (56). Authentic TAT migrates with an $R_f = 0.295$ in this system. Aminotransferase mRNA activity was expressed as the percent of total [³H] leucine incorporated into protein that was detected in the specific immunoprecipitate.

Isolation of Total Nuclear RNA

Fetal and neonatal liver nuclei were prepared by a modification of the citric acid procedure (57,58). The tissue was homogenized in two and one-half volumes of 1 mM MgCl₂, an equal volume of buffer A (100 mM citric acid, 1 mM MgCl₂) was then added and mixed with one

stroke. The homogenate was filtered through four layers of sterile cheesecloth and centrifuged at 300 x g for 10 minutes, at -10°C. The pellet was resuspended in 5 to 10 ml of buffer B (50 mM citric acid, 1 mM MgCl₂, 1.0% Triton X-100) using a small glass Dounce homogenizer. An equal volume of buffer C (0.5 M sucrose, 50 mM citric acid, 1 mM MgCl₂, 1.0% Triton X-100) was added and the mixture layered on top of 10 ml buffer D (0.32 M sucrose, 50 mM citric acid, 1 mM MgCl₂, 1.0% Triton X-100) in a 30 ml Corex tube, then centrifuged at 800 x g for 10 minutes, at -10°C. The nuclei were resuspended in a small volume (0.5 to 1.0 ml per 2 to 3 g tissue) of 25% glycerol, 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂ and stored at -20°C prior to the isolation of nuclear RNA.

The nuclei were lysed on ice in approximately 7 to 8 ml of 5 M guanidine thiocyanate (Kodak), 25 mM Tris-HCl, pH 7.5, 0.5% Na-N-lauryl sarcosinate, 0.1 M β-mercaptoethanol. Seven and one-half ml of that extract was layered on top of a 4 ml cushion of 5.7 M CsCl, 100 mM EDTA, pH 7.4 in an SW41 polyallomer tube. The samples were centrifuged at 36,000 rpm for 17 to 18 hours at 20°C (59). Under these conditions, the RNA is pelleted to the bottom of the tube. Each pellet was dissolved in 1.0 ml of 0.5 mM EDTA, 0.05% SDS by heating for 30 to 60 seconds at 65°C. The RNA was precipitated by the addition of Na-acetate to 0.23 M and two and one-half volumes ETOH.

Hybridot Analysis

Cytoplasmic poly(A) RNAs or total nuclear RNAs were bound to nitrocellulose sheets under conditions of high salt (20X SSC: 3 M

NaCl, 0.3 M Na-citrate) using the Bethesda Research Laboratory (BRL) hybridot manifold. Prior to spotting with RNA, the nitrocellulose sheets (BRL) were rinsed with H₂O and then soaked in 20X SSC for approximately 2 to 6 hours. After spotting the RNAs, the nitrocellulose sheet was baked in vacuo for 2 to 4 hours at 80°C. The sheets were prehybridized for 2 to 16 hours in a small volume of 50% deionized formamide, 1X hybridization buffer (5X SSC, 50 mM Na-phosphate, pH 6.5, 250 µg/ml sheared salmon sperm DNA, 0.02% each of bovine serum albumin (BSA), Ficoll and polyvinylpyrrolidone) at 42°C as described by Thomas (60). For each experiment, the appropriate amount of probe, 2 to 3 x 10⁶ cpm of T4 DNA polymerase labeled cDNA^{TAT}, was taken, denatured by heating at 100°C for 5 minutes, quick-chilled, added to an equal volume of 100% deionized formamide and then mixed with 50% formamide, 1X hybridization buffer to the final volume. This solution was then added to the pretreated nitrocellulose sheet for 18 to 24 hours hybridization at 42°C. After hybridization, unbound DNA was removed from the sheet by washing with 2X SSC, 0.1% SDS at 50°C, then with 0.2X SSC, 0.1% SDS at 42°C, repeating this cycle 2 to 4 times. The washed dot blot was exposed to Kodak X-AR5 film, with two Cronex intensifying screens at -70°C for one to two days. The hybridot films were analyzed by densitometric scanning.

T4 DNA Polymerase Labeling of Cloned cDNA^{TAT}

Purified cloned cDNA^{TAT} (Hae III/Hha I or Hind III/Ava II fragments) was generously made available by Kai-Lin Lee. The cDNA strand of the DNA duplex was preferentially labeled using T4 DNA

polymerase (61). The theory of the procedure is as follows. Nucleotides are excised from the 3' termini by the T4 enzyme in the absence of deoxynucleoside triphosphates (dNTPs). The addition of unlabeled dNTPs plus α -[^{32}P]-dCTP inhibits the exonuclease activity and initiates the resynthesis (filling-in) reaction. Thus the end product of the reaction is an intact, labeled duplex, suitable not only for hybridization experiments but also chromatographic studies. For each reaction, 0.2 to 0.5 μg of DNA were digested with T4 DNA polymerase (6.25 units enzyme/ μg DNA) in a buffer of 33 mM Tris-acetate, pH 7.9, 66 mM K-acetate, 10 mM Mg-acetate, 100 $\mu\text{g}/\text{ml}$ BSA, 0.5 mM dithiothreitol in 20 μl for 60 minutes, at 37°C. The synthesis reaction was initiated by the addition of dNTPs to 150 to 200 μM and 50 μCi α -[^{32}P]-dCTP (New England Nuclear NEG - 013H, > 2800 Ci/mM) in a final volume of 50 μl , and the mixture was incubated for 45 minutes, at 37°C. The reaction was forced to completion by the addition of unlabeled dCTP for a second 45 minute interval (total synthesis time: 90 minutes). The reaction was terminated by the addition of EDTA. The labeled DNA was separated from unincorporated α -[^{32}P]-dCTP by gel filtration chromatography over a 10 ml column of Biorad A.5m agarose beads in A.5m column buffer (10 mM Tris-HCl, pH 8.0, 80 mM NaCl, 0.2 mM EDTA). The DNA eluted in the void volume; those fractions containing labeled material were pooled and stored at 4°C. The specific activity of this material was greater than 1×10^7 cpm/ μg DNA.

Sucrose Gradient Analysis of Post Mitochondrial Supernatants

Perinatal and adult livers were homogenized in two volumes 0.25 M sucrose in PB and centrifuged to prepare a 27,000 x g supernatant fraction. To each sample, heparin was added to 0.5 mg/ml and Triton X-100 and Na-deoxycholate (DOC) to 1.0%. One-half ml was then layered on a linear 0.303 M to 1.375 M sucrose gradient in PB plus heparin with a one ml cushion of 1.8 M sucrose and centrifuged at 39,000 rpm for 4 hours, 15 minutes, at 4°C in an SW40 rotor (conditions modified from Geoghegan, et al. [49]). After centrifugation, each gradient was fractionated from top to bottom; 0.8 ml fractions collected, and the absorbance read at 254 nm.

To analyze the distribution of mRNA^{TAT} sequences, each gradient fraction was immediately denatured with an equal volume 12X SSC, 14.8% formaldehyde. An aliquot from each fraction was heated at 60°C for 20 minutes, then diluted 10-fold for application to nitrocellulose. This was a modification of a method described by White and Bancroft (62). Hybridization conditions were as described in Hybridot Analysis, this section. Gradient films were analyzed by densitometric scanning. For each gradient, the total number of integration units was calculated, and the fraction of mRNA^{TAT} sequences detected in each fraction is presented as a percentage of that total.

Results

Functional mRNA in Fetal Rat Liver

The routine immunoprecipitation assay for mRNA-directed synthesis of tyrosine aminotransferase was virtually negative when the

translation products of poly(A) RNA from late fetal livers were analyzed (47). It could be hypothesized that the inherent insensitivity of this heterologous in vitro system plus the significant fraction of globin mRNA present in fetal poly(A) RNA populations would mask the presumably low level of mRNA^{TAT} present. To circumvent these difficulties, extraordinary assay conditions were devised. Poly(A) RNA was isolated from a large number of litters by Mg⁺⁺-precipitation of polysomes and mRNA-protein complexes from 27,000 x g supernatant fractions followed by oligo(dT)-chromatography. Poly(A) RNAs greater than 18S were selected after centrifugation through sucrose gradients (Figure 1); a comparable preparation from adult liver was similarly treated to serve as the control. The partially purified poly(A) RNAs were translated in a 1.0 ml reaction, thus increasing by 10-fold over standard assay conditions the number of mRNAs to be assayed. With these modifications, functional mRNA^{TAT} was detected in fetal poly(A) RNA as seen in the electrophoretic profiles of immunoprecipitates shown in Figure 2. Given the usual correction for background radioactivity, the aminotransferase mRNA activity of the 18 and 19 day fetal sample was 0.019% of the total selected mRNA activity. Another experiment using these procedures and poly(A) RNA from 20 and 21 day fetal livers gave a mRNA^{TAT} activity of 0.02% of the total. Similarly analyzed, the adult level was 0.27%. Therefore, in good agreement with the observed 10 to 15-fold difference in specific enzyme activities between fetal and adult livers, this analysis showed that mRNA^{TAT} activity is

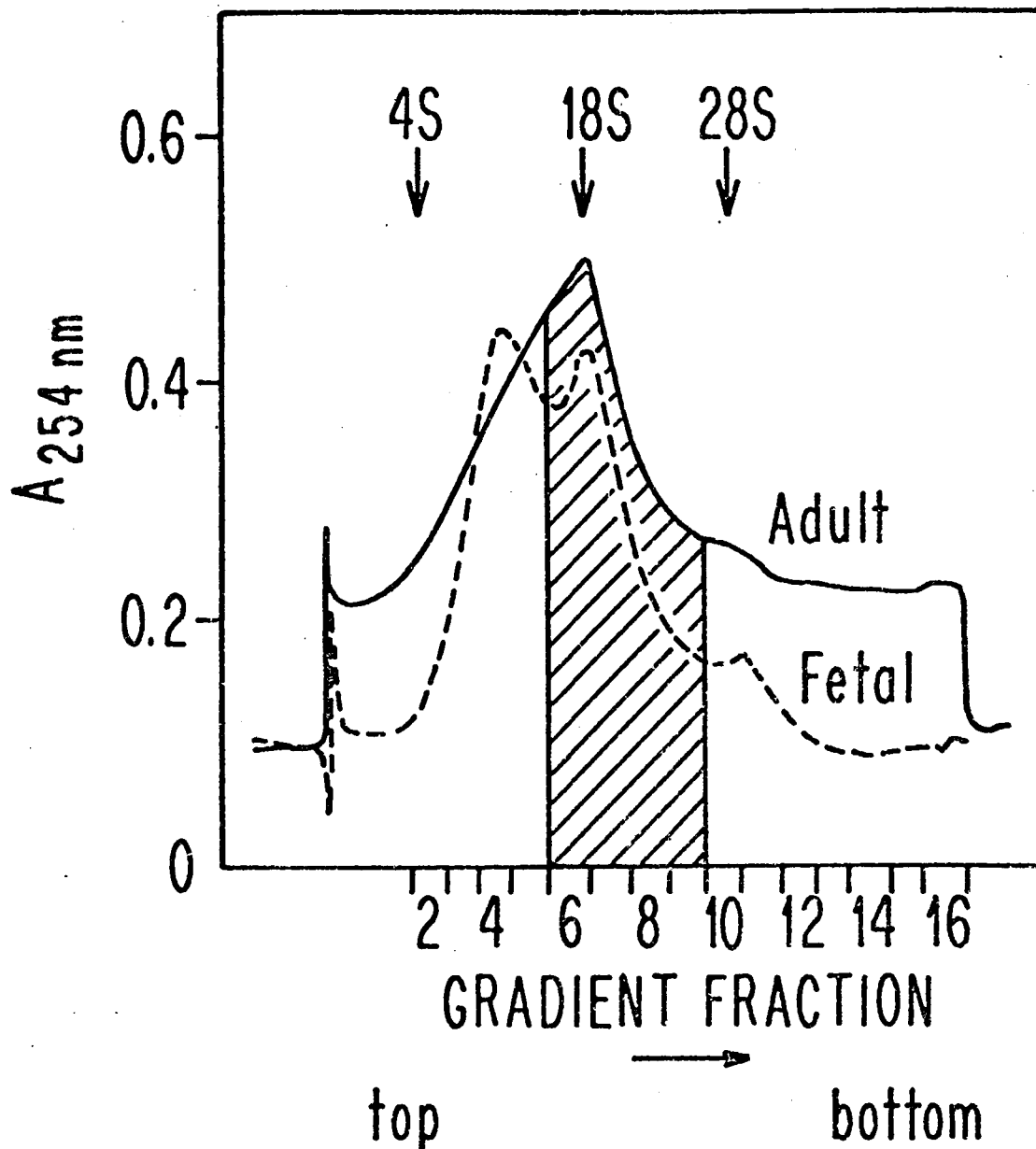


Figure 1. Selection of Large mRNAs for Translation. Adult and 18 and 19 day fetal poly(A) RNAs were partially purified by centrifugation through 5 to 20% sucrose gradients as described in the text. For each sample, fractions 6 through 9 were pooled and assayed for mRNA^{TAT} activity by translation in a reticulocyte lysate system.

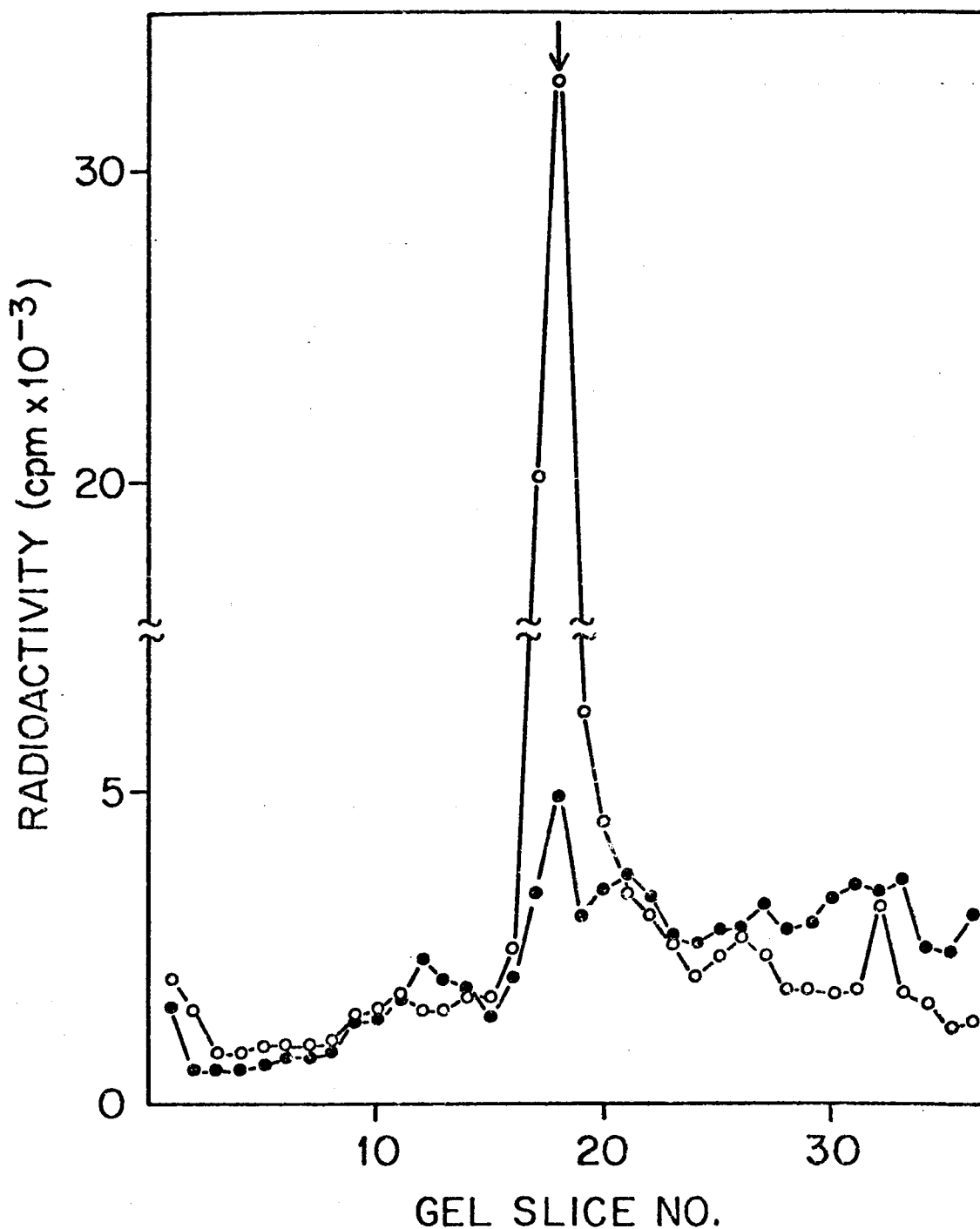


Figure 2. Electrophoretic Analysis of Immunoprecipitates of In Vitro Translation Products Directed by Size-Selected Poly(A) RNAs. Large poly(A) RNAs from fetal (●—●) and adult (○—○) livers were translated in vitro. Immunoprecipitates were purified, subjected to electrophoresis in SDS-polyacrylamide gels, and the gels sliced and counted. Authentic aminotransferase subunits migrate with an R_f of 0.295 (arrow).

indeed present in fetal rat liver, but at levels at least 10-fold less than in the adult.

Titration of Hybridizable RNA Sequences

Several technological advances within the past two years made this analysis possible. First, a cloned cDNA, specific for tyrosine aminotransferase sequences, was prepared (manuscript in preparation). Then, a method was worked out using T4 DNA polymerase to label that DNA to a high specific activity (61). The development of hybridot methodology (60) as an alternative to solution hybridization to quantitate the relative number of sequences made the analysis of multiple samples easier and more direct. Finally, the use of densitometric scanning facilitated the interpretation of the hybridot autoradiographs by assigning a number proportional to spot intensity.

Perinatal poly(A) RNAs were bound to nitrocellulose, probed with labeled cDNA^{TAT}, and exposed to X-ray film as described. Figure 3 illustrates the kind of data one obtains from the dot hybridization technique. The film presented in Figure 3 was scanned; Figure 4 presents a plot which compares integration units detected to the concentration of RNA applied for five of the samples. Within a linear scanning range, the number of integration units plotted was proportional both to the amount of RNA available and to the extent of hybridization detectable. To account for varying ranges of linearity relative to RNA concentration for different time points, the best fit line was calculated by linear regression for each sample. The relative number of sequences were quantitated by comparing for each

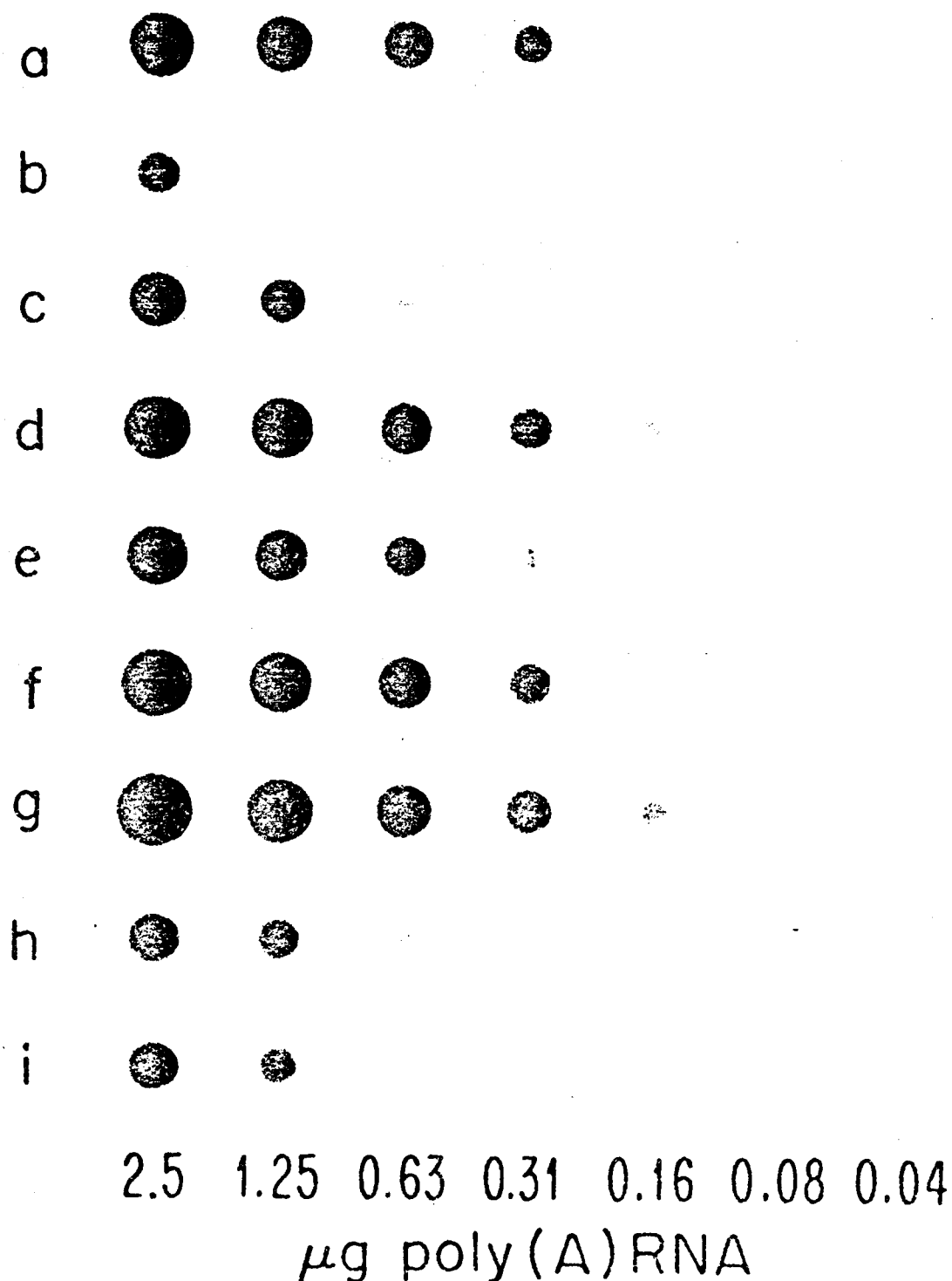


Figure 3. Hybridot Analysis of mRNA^{TAT} Sequences in Perinatal Rat Liver. Cytoplasmic poly(A) RNAs were isolated and bound to nitrocellulose sheets. Following hybridization with labeled cDNA^{TAT}, the extent of hybridization was detected by autoradiography. Adult, a; 19 and 20 day fetal, b; 21 day fetal, c; newborn at birth, d; 6 hours after birth, e; 12 hours, f; 19 hours, g; 24 hours, h and i.

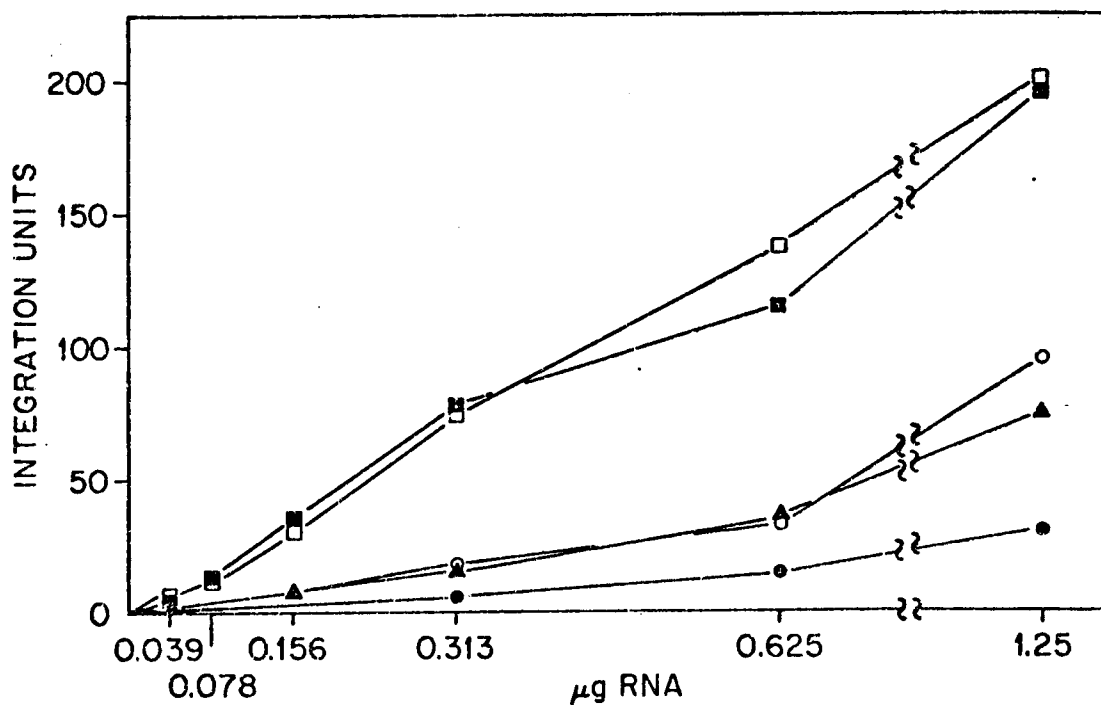


Figure 4. Integration Units Derived from Densitometric Scanning are Proportional to RNA Concentration. The hybridot presented in Figure 3 was scanned using a Zeineh soft laser scanning densitometer. The integration units for each spot are plotted against RNA concentration for each sample. The data were analyzed as described in the text. 19 and 20 day fetal (●—●); 21 day fetal (○—○); newborn at birth (■—■); 12 hours after birth (□—□); 24 hours (▲—▲).

sample the number of integration units for 0.5 μ g of RNA, as determined from the best-fit line.

The results of a large number of samples and experiments are summarized in Figure 5. The lowest level of sequences was titrated from 19 and 20 day fetal samples. This level was set as one to establish a baseline for comparison and further discussion. The relative number of sequences increased two-fold the day prior to birth and remained constant until parturition. This increase in sequence concentration (although consistent over the last day and a half of gestation) represents a comparison of the means from each time point sampled; the difference was found not to be statistically significant ($\alpha=0.05$). In poly(A) RNA from newborn liver, hybridizable amino-transferase mRNA sequences were at the adult level. The increased number of sequences was unexpected; therefore this time point was sampled many times and the initial observation was confirmed. The level of sequences remained statistically ($\alpha=0.05$) constant during the first 12 hours after birth. Sequences declined thereafter to 24 hours, the last neonatal time point analyzed.

Developmental activation of tyrosine aminotransferase has now been described in terms of enzyme and mRNA^{TAT} activities (44,47) as well as by titration of hybridizable sequences, as shown in Figure 6. Most striking is the accumulation of sequences in the newborn cytoplasm without a concomitant increase in either enzyme or mRNA^{TAT} activity. It is of note that the sequences are without function both in the hepatocyte and in the reticulocyte lysate system.

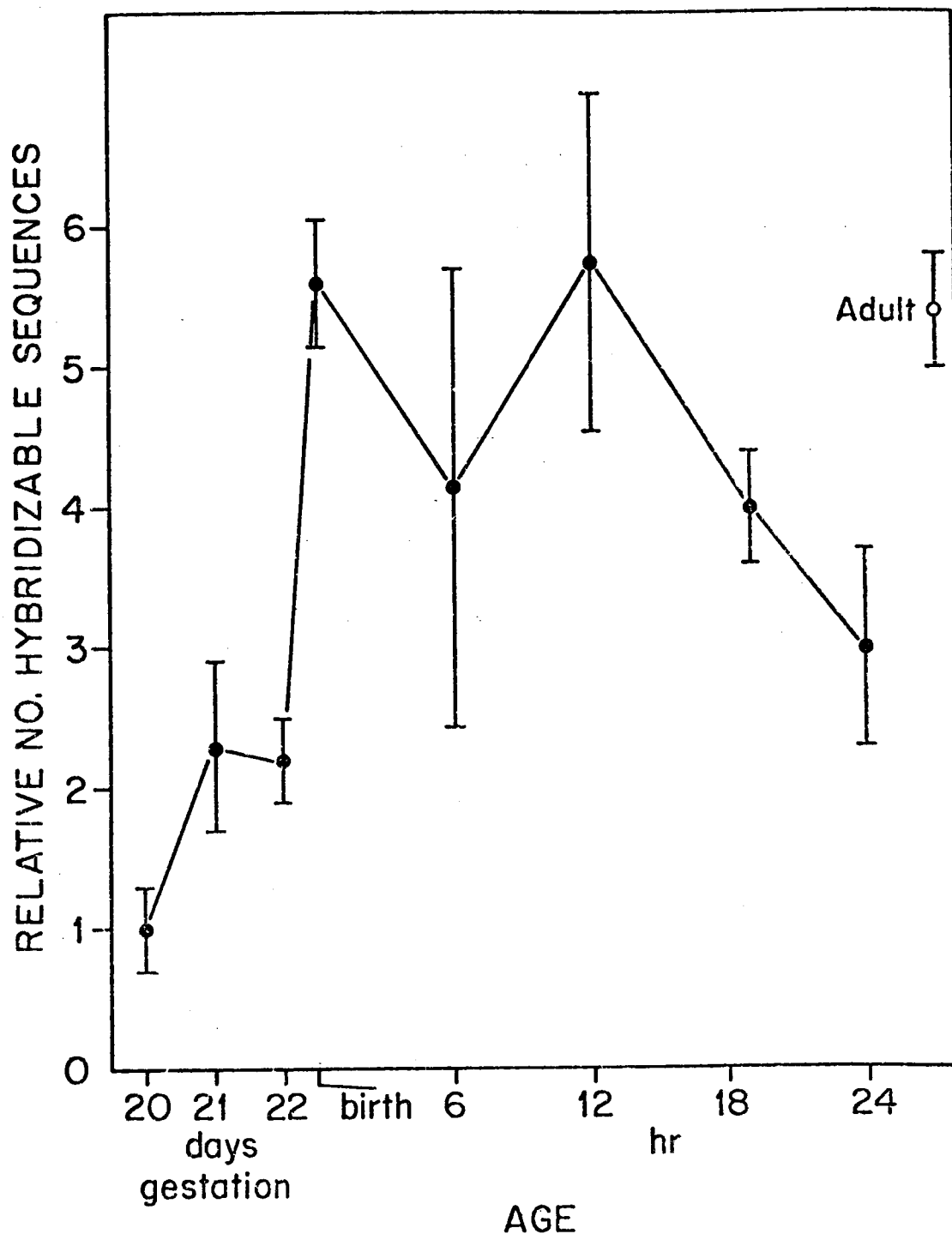


Figure 5. Accumulation of Hybridizable Cytoplasmic mRNA^{TAT} Sequences During Development. Hybridot films were analyzed by densitometric scanning and quantitated as described in the text. All perinatal and adult samples tested have been related to the mean value of 20 day fetal RNAs. Each point represents the mean $\pm$ S.E. for multiple determinations of 2 to 4 samples at each time point.

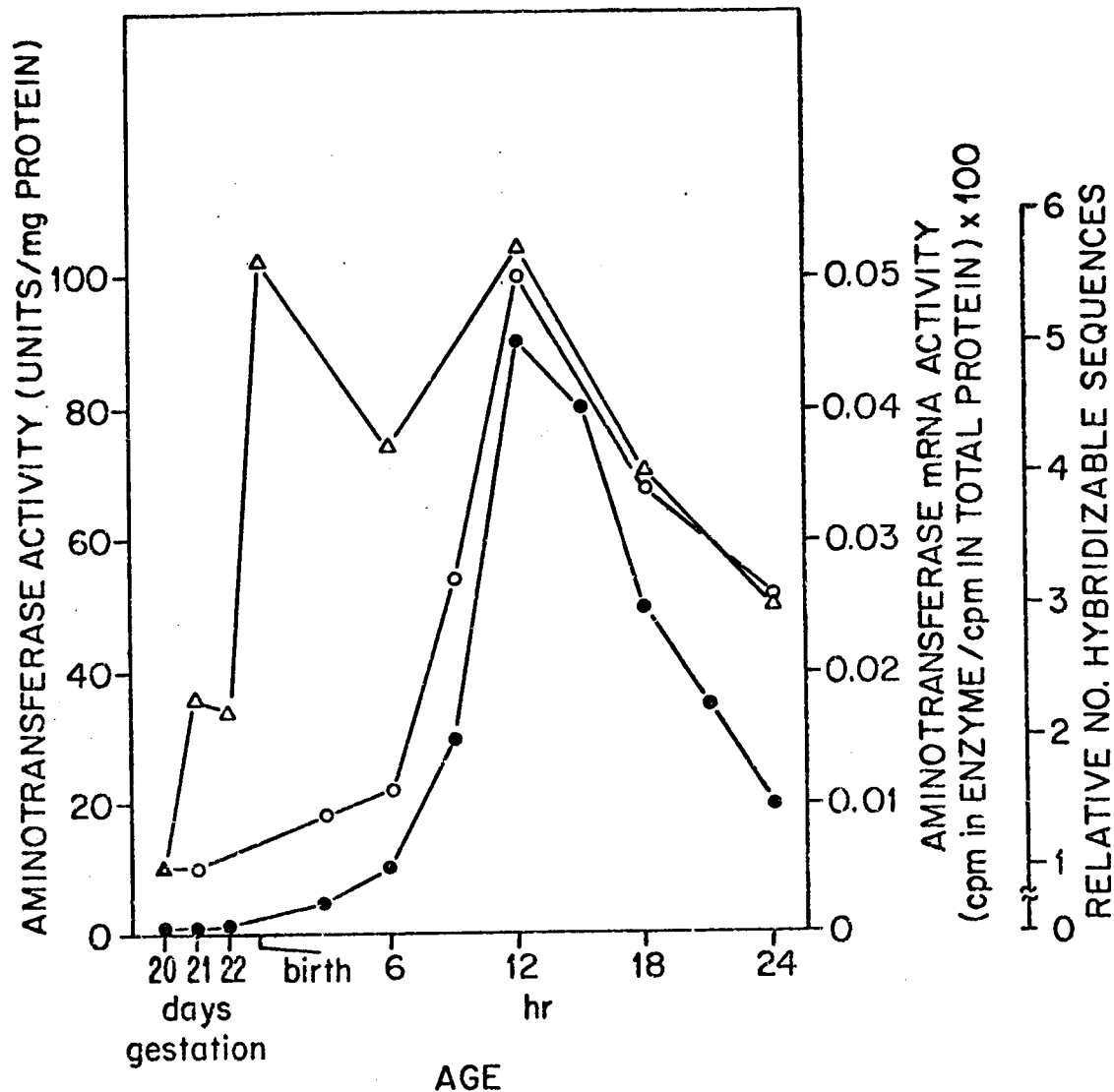


Figure 6. Time Course of the Developmental Activation of Tyrosine Aminotransferase Expression. Three parameters of TAT expression have been analyzed throughout the perinatal period. The enzyme was assayed in the 27,000 x g supernatant fraction of liver homogenates (●—●). Cytoplasmic poly(A) RNA was isolated. The mRNA-directed *in vitro* translation products were immunoprecipitated to quantitate mRNA^{TAT} activity at each time point (○—○). These data are replotted from Perry, *et al.* (47). Hybridizable mRNA^{TAT} sequences were quantitated as described in Figure 5 (△—△).

The increase in cytoplasmic transcripts seen just before and at birth are likely to be the consequence of increased gene transcription. As an approach to determining this, nuclear transcripts were measured since they would be expected to change in parallel if transcription were responsible for the increase in cytoplasmic sequences.

Titration of nuclear transcripts by the hybridot method showed that hybridizable nuclear RNA sequences did indeed increase prior to birth and in newborns. Furthermore, the sequences accumulated to the same extent and with the same kinetics as the cytoplasmic mRNA^{TAT} sequences (Figure 7). From this analysis, it would appear that the increase in cytoplasmic sequences is the consequence of an increased level of TAT transcription. Direct measurement of this parameter would involve analysis of run-off transcripts in isolated nuclei. Many attempts have been made to isolate "transcription-grade" nuclei from fetal liver, but to date, suitable nuclei have not yet been obtained.

Sequence Distribution in Post-Mitochondrial Supernatant Fractions

The accumulation of cytoplasmic transcripts for a significant period of time prior to their functional appearance, ascertained either by enzyme activity in vivo or by translational capacity in vitro, argues strongly for a post-transcriptional point of regulation, probably at the level of translation. In this case, one could hypothesize that the translationally inactive sequences might be sequestered in a nonpolysomal compartment of the cytoplasm.

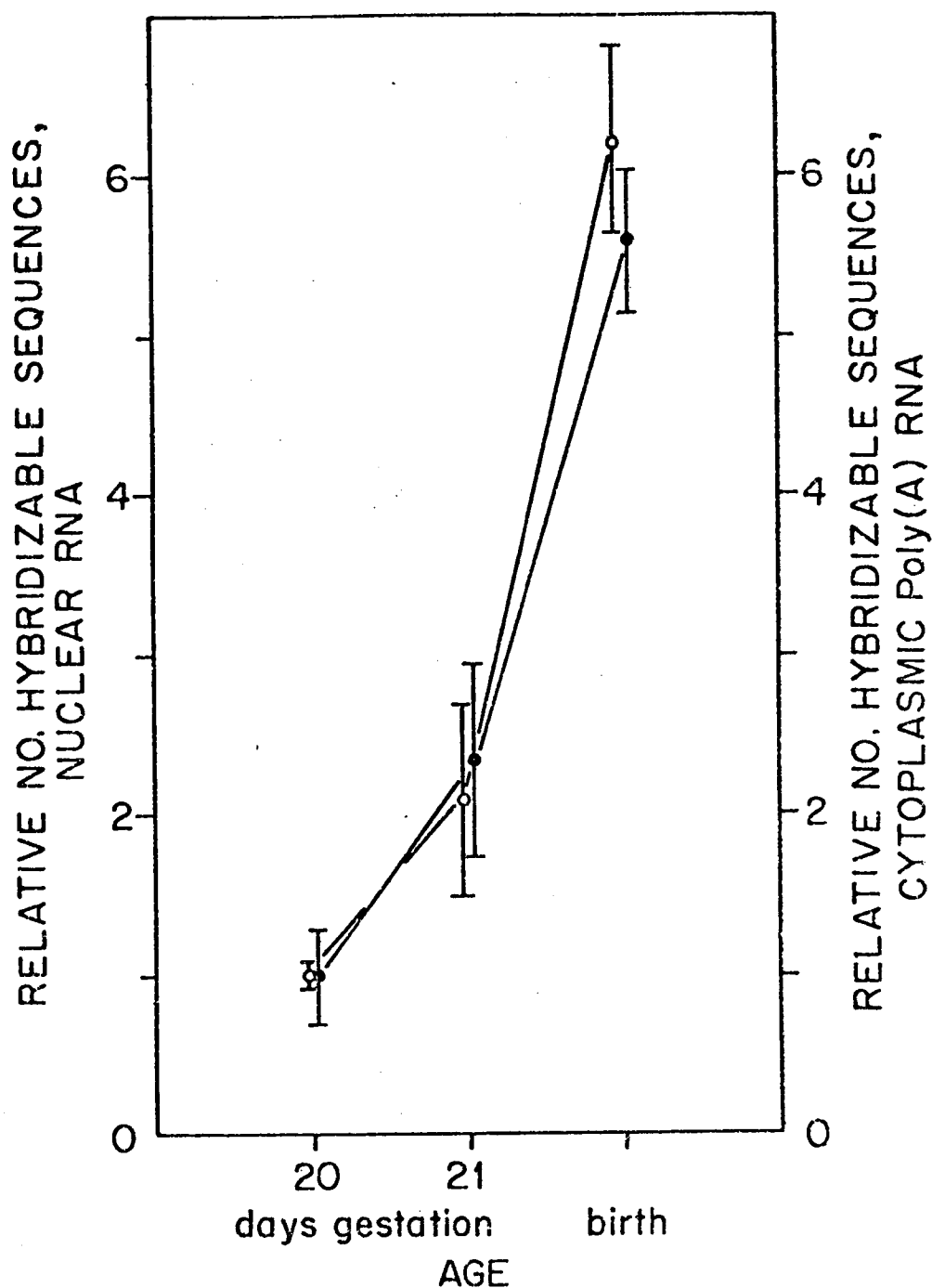


Figure 7. Hybridizable Nuclear RNA Sequences Increase Immediately Prior to Birth. Total nuclear RNA (○—○) was isolated by lysing purified fetal and newborn nuclei in 5 M guanidine thiocyanate extraction buffer and pelleting the RNA by centrifugation through 5.7 M CsCl₂, 100 mM EDTA. Analysis and quantitation were done as described in Figures 3 and 4. Each point represents the mean $\pm$ S.E. for multiple determinations of three individual samples at each time point. Cytoplasmic (●—●) sequence measurements are presented for comparison.

In order to test this hypothesis, the following experiment was done. Post-mitochondrial supernatants from fetal, newborn, and adult rat liver homogenates were treated with heparin, Triton X-100 and DOC and analyzed after centrifugation through 0.303 M to 1.375 M sucrose gradients. Two sets of information were generated from each gradient, an absorbance profile and a pattern of the hybridizable mRNA^{TAT} sequences. The most dramatic observation from this set of experiments was the diminution in large polysomes with a concomitant increase in monosomes in the extracts from newborn livers relative to either the fetal or the adult samples. The differing absorbance profiles of each timepoint are superimposed on essentially identical patterns of mRNA^{TAT} sequence distribution; the majority of the sequences appear to be in the polysome fraction, as shown in Figure 8. Therefore, in spite of widely varying RNA-protein complex compartments, the array of mRNA^{TAT} sequences appeared to remain constant.

Discussion

Selection and translation of partially purified fetal poly(A) RNAs established unequivocally that active mRNA^{TAT} can be detected in late-term fetal liver. This is contrary to the report of Ruiz-Bravo and Ernest (63), who used only conventional translation techniques. Together with demonstration of authentic enzyme activity by Perry, et al. (47), these results establish that the TAT gene is being expressed in late term fetuses, albeit at very low levels relative to the adult.

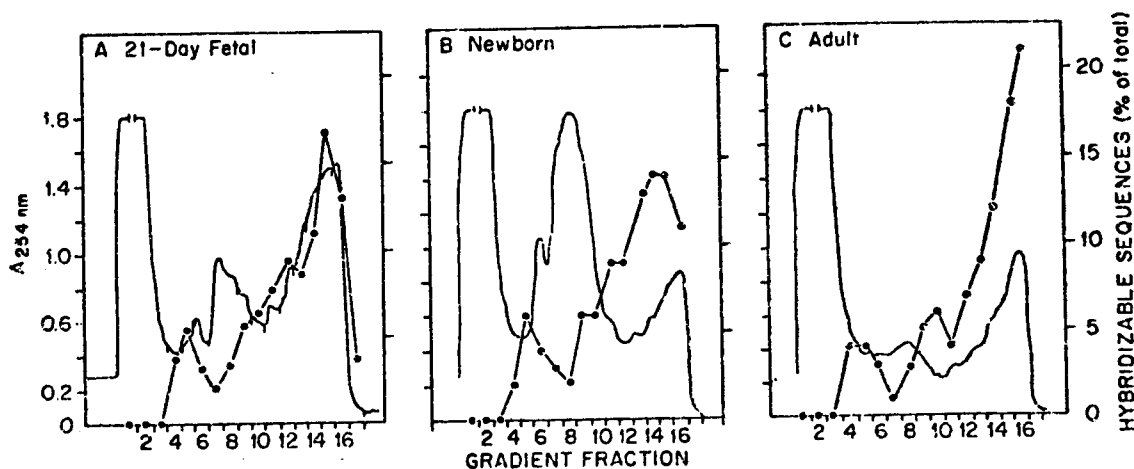


Figure 8. Sucrose Gradient Analysis of mRNA^{TAT} Sequence Distribution in Post-Mitochondrial Supernatants. Equal volumes of fetal, newborn and adult post-mitochondrial supernatant fractions were layered onto 0.303 M to 1.375 M sucrose gradients and centrifuged as described in the text. Each gradient was fractionated and the absorbance at 254 nm was recorded. Each gradient fraction was immediately denatured with an equal volume 12X SSC, 14.8% formaldehyde. An aliquot from each fraction was heated at 60°C for 20 minutes then diluted 10-fold prior to application onto nitrocellulose. Hybridization conditions are as outlined in Figure 3.

Messenger RNA sequence titration of cytoplasmic poly(A) RNAs revealed several interesting and important aspects of developmental gene activation. The lowest level of sequences were found in 19 to 20 day fetal livers. This is in agreement with the low levels of functional gene products. Sequences increased rapidly in the last days prior to birth and are at or greater than adult levels in the newborn. Owing to the heterogeneity of the perinatal liver cell population, precise comparisons are not meaningful. This increase in hybridizable sequences is likely due to an increased rate of TAT transcription. In accord with this concept, nuclear RNAs were found to increase to the same extent and with the same kinetics as the cytoplasmic sequences. This analysis does not preclude changes in mRNA degradation or the processing of transcripts as possible mechanisms controlling the number of sequences. The means and the methods to distinguish between these possibilities have not yet been developed.

The observation that hybridizable sequences increase well before any change in functional mRNA is direct evidence for post-transcriptional control superimposed on the presumed transcriptional change responsible for the increased number of transcripts. Dot blot data are limited in that no structural information concerning the hybridizable sequences is presented. There are reports in the literature (64,65) of differing mRNA species during development which code for the same gene product. Northern analysis of fetal and newborn poly(A) RNAs (data not presented) do not show any difference in size, only in

quantity (and extent of degradation) of the hybridizing species. Thus, at this point, no structural feature can be evoked to account for the degree of translational capacity of the perinatal mRNA^{TAT} sequences.

Analyses designed to determine if mRNA^{TAT} is nonfunctional owing to sequestration in a nonpolysomal compartment indicate that this is not the case. The nonpolysomal RNA-protein complex compartment is one of great flux. The mRNAs present in this compartment can include those just transported from the nucleus, those bound with translational inhibitors (either protein or RNA species) and those in the process of degradative turnover (66). Thus, it must be recognized that the potential for these experiments to yield artifactual results is large; i.e., the nonfunctional mRNA may be present in an unusually labile configuration and would not be detected. Further work is needed, and is in progress, to clarify this and to define the factor(s) responsible for the inability of those transcripts to be translated.

CHAPTER III

ANALYSIS OF THE EFFECT OF 5-AZACYTIDINE ON HEPATIC DIFFERENTIATION

Introduction

A description of normal TAT developmental activation in terms of enzyme and mRNA^{TAT} activities and of TAT transcript accumulation now exists. It can be inferred from the data presented that activation of TAT expression is primarily the consequence of an increased rate of TAT gene transcription. Now the question moves one step back--what is the molecular mechanism, what happens to the TAT gene/chromatin structure so that transcription is increased?

In several recent studies, site-specific DNA demethylation has been proposed as a key mechanism in specific gene activation occurring during differentiation. 5-Azacytidine is a potent inhibitor of DNA methylation but has several other biological effects (20). The mechanism of the hypomethylating effect is not known, although models have been proposed (28). In cell culture, treatment with this compound has induced cellular differentiation (22-24), reversed X-chromosome inactivation (21), and modulated the expression of specific genes (26). Treatment of a β^+ -thalassemia patient with 5-azaC reactivated fetal β -globin gene expression (27).

Since the activation of TAT expression is a model for acquisition of liver-specific functions by the developing hepatocyte population, it was of interest to determine if the extent or nature of specific

DNA methylations could serve a regulatory function in TAT development. Thus, by treating fetuses in utero with 5-azaC, as a hypomethylating agent, I asked whether DNA methylations may be involved in the activation of TAT expression during normal hepatocellular differentiation.

Experimental Procedures

Animals

Sprague-Dawley rats were bred and pregnancies timed as described in Chapter II. All injections were done late in the twentieth day of gestation. 5-Azacytidine (Sigma) was dissolved in sterile 0.15 M NaCl at the appropriate concentrations and injections of 100 μ l were made intraperitoneally to individual fetuses of anesthetized, laparotomized dams. The abdomen was then sutured closed. At the appropriate times following injection, the dams were again anesthetized and the fetuses taken and killed by decapitation. Controls were age-matched fetuses, either untreated, or administered similar concentrations of cytidine or cytosine arabinoside (Sigma), or of 6-azacytidine (PL Biochemicals) in the same fashion as 5-azaC was given.

Enzyme Assays

For determination of tyrosine aminotransferase enzyme levels, 12,000 x g or 27,000 x g supernatants of liver homogenates were prepared and then assayed by a modification of the method of Briggs (67); the enzyme unit is the amount catalyzing the formation of 1 nmol product per minute. Phosphoenolpyruvate carboxykinase (E.C. 4.1.1.32,

PEPCK) activity was measured in 105,000 x g supernatant fractions of fetal or adult liver homogenates. The homogenates were prepared by homogenizing freshly excised tissue in five volumes of a buffer containing 50 mM Hepes, pH 7.6, 100 mM KCl, 1 mM EDTA, 1 mM DTT. The homogenates were filtered, centrifuged to prepare first a 27,000 x g supernatant, which was then centrifuged at 34,000 rpm for 60 minutes in a type 50 rotor at 4°C to prepare the 105,000 x g supernatant. The assay determines the IDP-dependent incorporation of NaH [¹⁴C] CO₃ into oxaloacetate, with malic dehydrogenase (NADH) driving the reaction (and labeled product) to malate (method courtesy Wesley D. Wicks, from 68); the enzyme unit is the amount catalyzing the formation of 1 nmol product per minute. Glucose-6-phosphatase (E.C. 3.1.3.9) and tryptophan dioxygenase (E.C. 1.13.1.12) were assayed as described by Burch, et al. (69), and Schutz and Feigelson (70), respectively. Protein content of the supernatant fractions was estimated using Biorad protein reagent, with BSA as the standard.

Polyacrylamide Slab Gel Electrophoresis

Slab gels containing 13% (or 10%) acrylamide, and 0.35% (or 0.27%) bis-acrylamide were prepared with 3% acrylamide stacking gels, following the procedure of Laemmli (71). Protein samples (from 105,000 x g supernatant fractions or in vitro translation assays) were electrophoresed through the stacking gel at 30 mA (constant current), then at 100 V (constant voltage) through the resolving gel until the dye front reached the edge. The gel was then fixed and stained

overnight in 0.05% Coomassie blue, 45% EtOH, 10% acetic acid, and then destained in 5% methanol (MeOH), 7% acetic acid. The destained gels were analyzed by densitometric scanning. If the gel was to be analyzed by fluorography, it was fixed first in 10% TCA, 10% acetic acid, 30% MeOH for 4 to 5 hours, and then soaked overnight in 10% acetic acid, 30% MeOH. Next, the gel was treated for 30 minutes with Enlightening (New England Nuclear), dried under vacuum for one hour, then wrapped in Saran and exposed to X-ray film with two Cronex intensifying screens at -70°C for one to two days.

Histology

Freshly excised livers from treated and untreated 21 day fetal rats (0.15 g liver wet weight) were fixed for several days in 10 ml buffered formalin prior to embedding, sectioning, mounting and staining. Paraffin sections were stained with hematoxylin and eosin to demonstrate tissue organization. Specific histochemical stains for glycogen (periodic acid-Schiff reagent, PAS) and lipid (Sudan black and/or oil red O) content were also performed.

Electron Microscopy

Freshly excised fetal rat livers from treated and untreated 21 day fetuses were immediately fixed in modified Karnovsky's fixative (2% glutaraldehyde, 2.5% paraformaldehyde, 0.1 M phosphate buffer) and post-fixed in 1% osmium tetroxide. The tissue was dehydrated in graded ethanols and then embedded in Epon 812. Pale gold sections were stained with uranyl acetate and lead citrate and examined with a

Siemens 1A electron microscope; low magnification (2,000X) survey photos were taken.

Other Procedures

Preparation of poly(A) RNA, dot hybridization analysis, in vitro translation assay and immunochemical titration, and sucrose gradient fractionation of RNA were carried out as described in Chapter II.

Results

Activation of Tyrosine Aminotransferase

I have found that treatment of 20 day fetuses in vivo with 5-azaC fully activates expression of the TAT gene.

Activation of aminotransferase activity, the first parameter studied, was strongly dose-dependent (Figure 9). At the lowest concentrations tested (100-200 µg/fetus) there was little effect on enzyme activity. At the optimal dose (400 µg/fetus) aminotransferase specific activity increased about 70-fold in 18 hours. Under these conditions survival was always greater than 50%. If the dose was increased to 500 µg, survival decreased to unacceptable levels and induction was reduced in the surviving fetuses. To approach the question of whether hypomethylation of DNA may be implicated in the in vivo activation of TAT expression by 5-azaC observed, rat fetuses were treated with unmodified cytidine and with the toxic analogs cytosine arabinoside or 6-azacytidine; none of these compounds affect the methylation of DNA. Results of these control experiments show that these agents are not capable of inducing the dramatic increase in

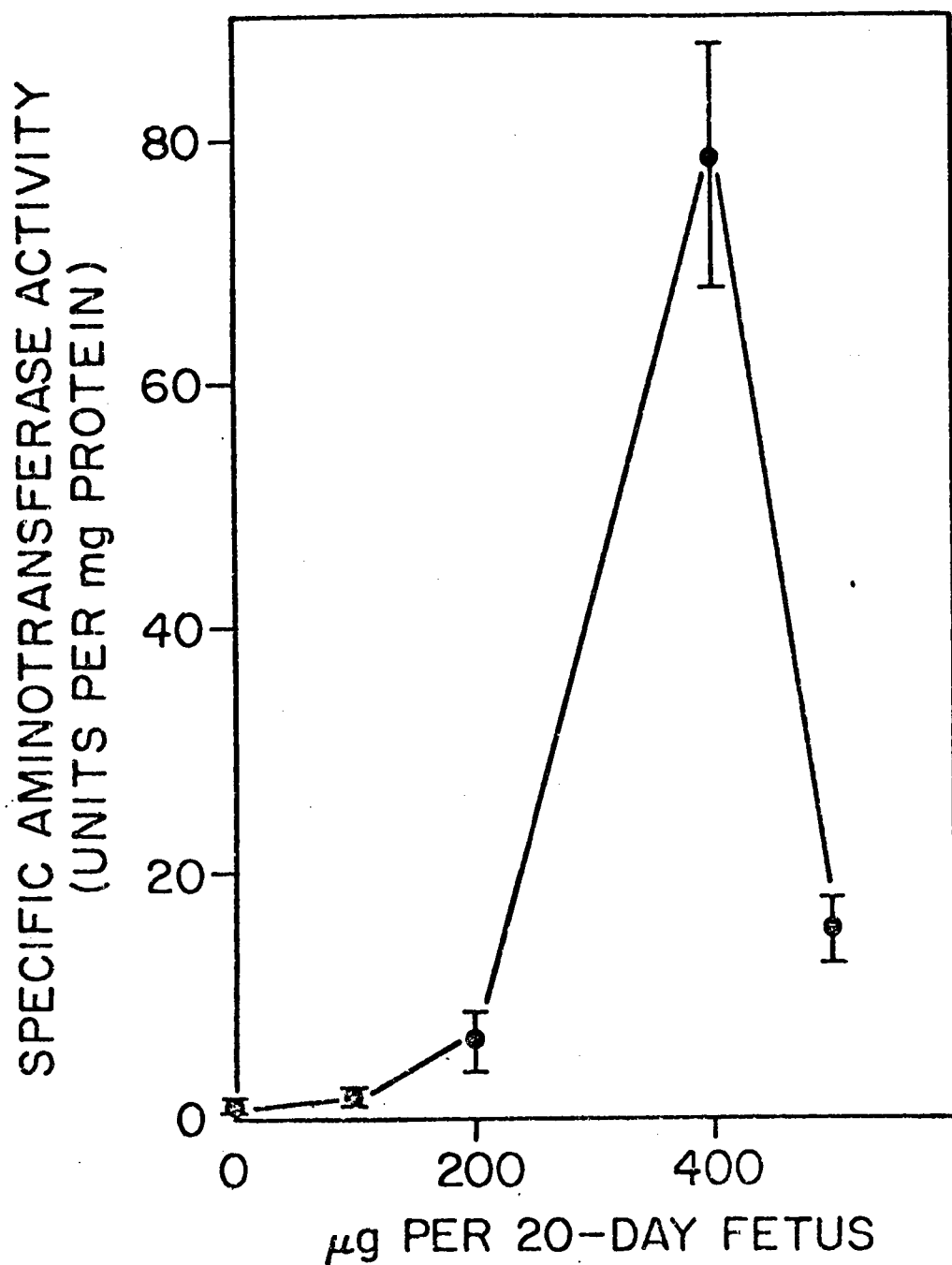


Figure 9. Dose Response of Tyrosine Aminotransferase Activity. Fetuses were treated with increasing doses of 5-azacytidine. All measurements were made 18 hours after treatment. The enzyme was assayed in 12,000 x g supernatant fractions of liver homogenates. Each point represents the mean $\pm$ S.E. for a minimum of three samples at each concentration tested.

expression effected by 5-azaC. The 6-aza analog did elicit a slight increase in aminotransferase activity in some fetuses but the effect was quite inconsistent; cytidine and cytosine arabinoside were without effect (Table I).

A number of experiments in which the time course of the appearance of the various parameters of TAT gene expression were measured, following an optimal dose of 5-azaC, are summarized in Figure 10. There is a lag time of about nine hours before an appreciable increase in enzyme activity is detected, followed by an abrupt and rapid increase in this parameter to very high levels (78.6 ± 10.33 units/mg protein) after 18 hours of treatment. This level is nearly five-fold above the steady-state level in adult livers (~ 16 units/mg protein). Toxicity of the drug became increasingly apparent at treatment times greater than 18 hours, so I have not extended these experiments beyond that time. In normal development, the postnatal activation of TAT expression is accompanied by acquisition of the capacity to respond to induction by glucocorticoids (44,47). In an effort to determine if the precocious activation by 5-azaC also confers this capacity, hydrocortisone (5 mg/100 g body weight) was given either intraperitoneally or intravenously to dams carrying treated fetuses, 13 hours after the drug and 5 hours before the fetuses were taken for analysis. The hormone did not further increase enzyme levels at 18 hours (54.8 ± 3.7 units/mg protein).

Like the development of enzyme activity following administration of 5-azaC, there is a lag period of about nine hours before an

TABLE I

Effects of Cytidine and its Analogs on Tyrosine Aminotransferase
Expression in Livers of Fetal Rats

Treatment	Aminotransferase Activity* (Units/mg Protein)
None	0.78 $\pm$ 0.11
Cytidine	0.52 $\pm$ 0.04
Cytosine arabinoside	1.06 $\pm$ 0.06
6-Azacytidine	2.56 $\pm$ 1.96
5-Azacytidine	78.61 $\pm$ 10.33

*Data are the mean $\pm$ S.E. for a minimum of three or four individual determinations made on pooled livers of three to four fetuses in each group. Doses of cytidine and its analogs were 400 μ g/fetus. Treatment time was 18 hours.

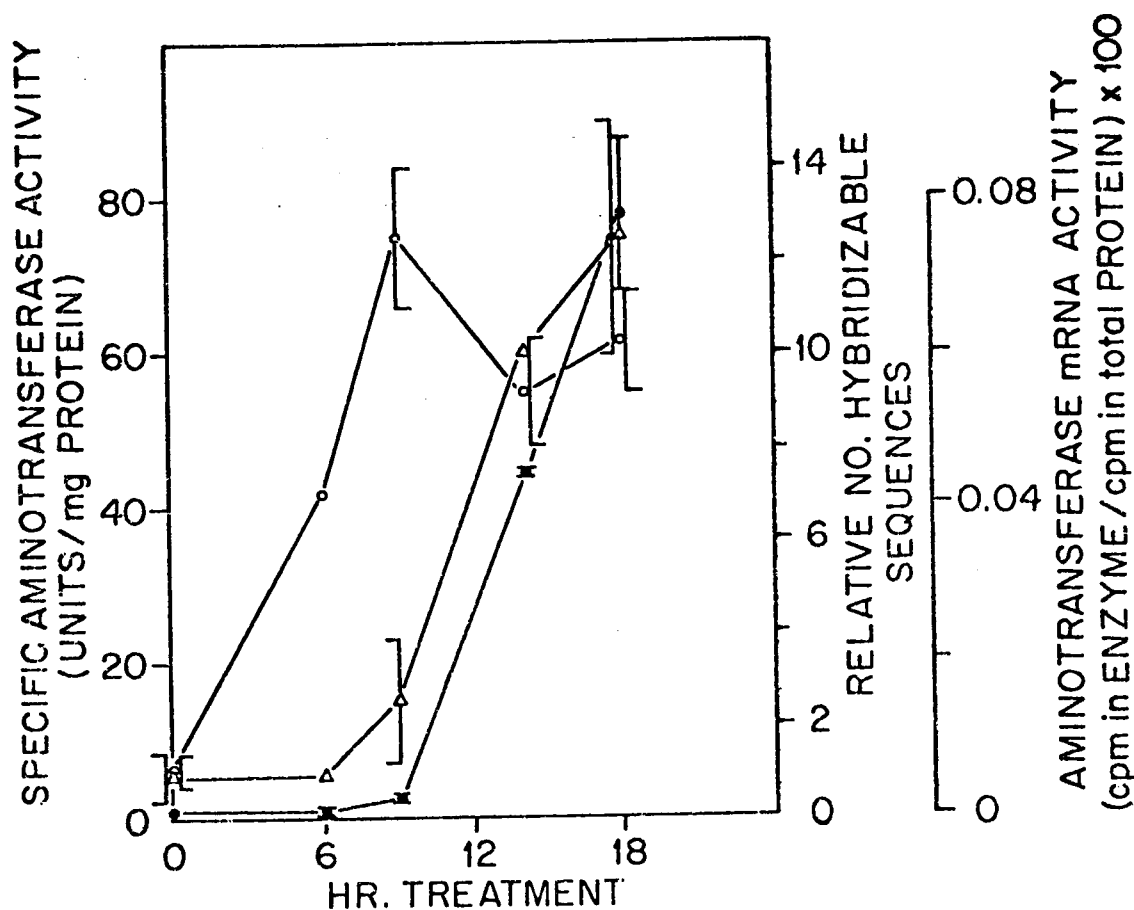


Figure 10. Time Course of Activation of Tyrosine Aminotransferase Expression. Fetuses were treated in utero with 400 μ g 5-azacytidine each. Three parameters of TAT expression were analyzed at each time point. The enzyme was assayed in the 27,000 x g supernatant fraction of liver homogenates ($\bullet$ — $\bullet$). Cytoplasmic poly(A) RNA was isolated. The mRNA-directed in vitro translation products were immunoprecipitated to quantitate $mRNA^{TAT}$ activity at each time point (Δ — Δ). Hybridizable $mRNA^{TAT}$ sequences were quantitated by hybridot analysis and densitometric scanning ($\circ$ — $\circ$). The data represent the mean $\pm$ S.E. for multiple determinations of 2 to 4 samples at each time point for all parameters assayed. The points without error bars represent analysis of a single sample.

increase in mRNA^{TAT} translational activity is detected. Following 18 hours treatment, the mRNA^{TAT} activity had increased 15-fold above untreated fetal values (~0.005%) to 0.075% of the total protein synthesized in the reticulocyte lysate system.

Hybridot analysis of cytoplasmic poly(A) RNAs detected a rapid accumulation of hybridizable sequences following 5-azaC treatment (Figure 11). The level of sequences increased seven-fold after six hours of treatment, peaking after nine hours at a level 12.5 times greater than that of the untreated 20 day fetus. As in normal development, once the sequences have peaked in quantity, there is a transient decline in their number (9.2 fold induced at 14 hours), followed by an increase after 18 hours (10.2 fold level of induction). The apparent decline in the concentration of sequences assayed at 14 or 18 hours relative to that concentration at 9 hours following treatment represents a comparison of means. The differences are not statistically significant at $\alpha=0.05$.

The time course of the appearance and functional activity of TAT gene products induced by 5-azaC closely mimics the pattern seen during normal development activation (Figure 6, page 26). Again, hybridizable mRNA^{TAT} sequences accumulate prior to the concerted acquisition of aminotransferase enzyme and mRNA activities.

Effects on Other Components of Hepatic Differentiation

Hepatic differentiation is influenced by 5-azaC in a more profound fashion than simply the activation of this single enzyme. In the course of the experiments described above, I noted that the gross

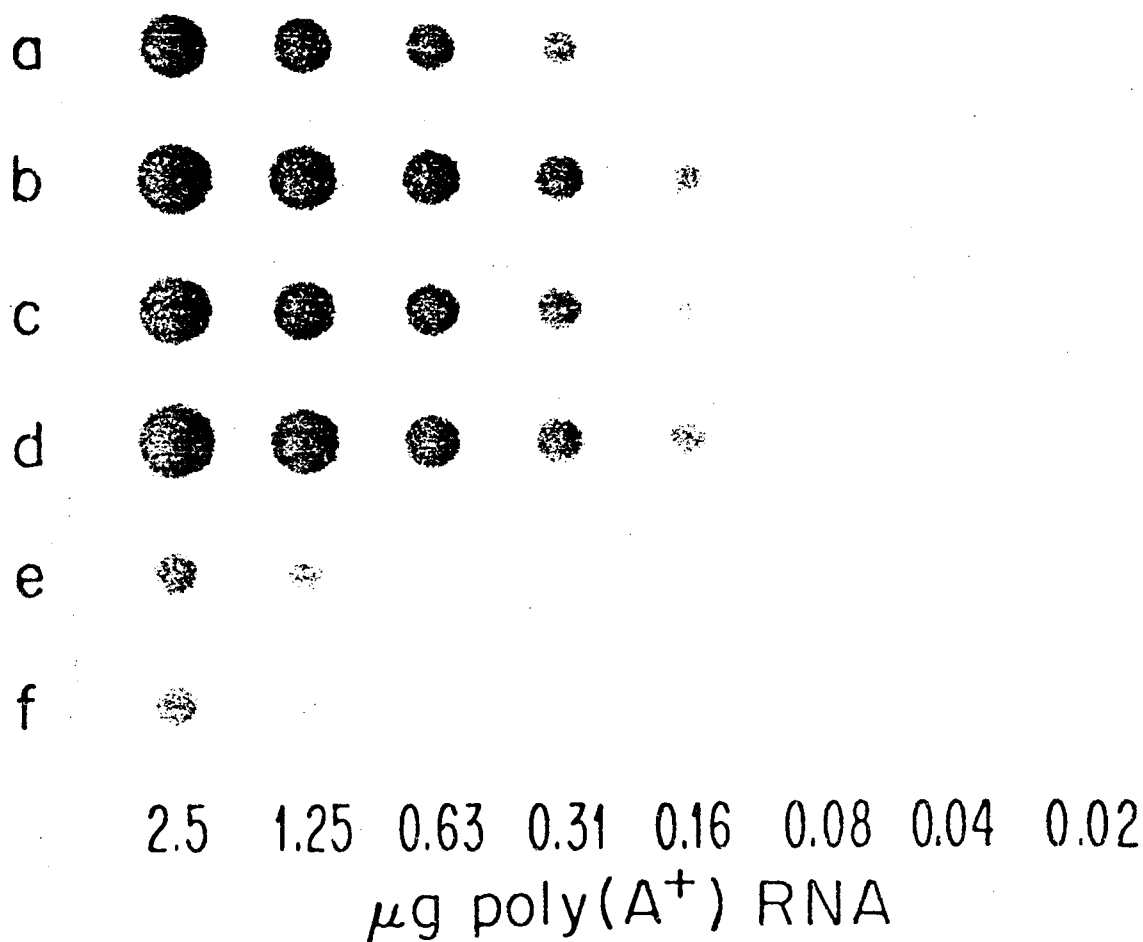
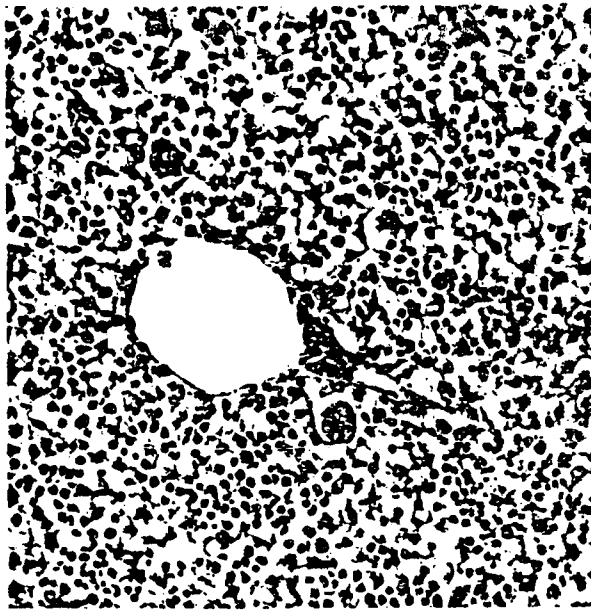


Figure 11. Hybridot Analysis of mRNA^{TAT} Accumulation Following 5-Azacytidine Treatment. Cytoplasmic poly(A) RNAs were isolated, bound to nitrocellulose sheets, and detected by autoradiography. Six hours following treatment, a; 9 hours, b; 14 hours, c; 18 hours, d; 19 day fetal, e; 19 and 20 day fetal, f.

appearance of the fetal liver was markedly altered 14 or 18 hours after treatment with the drug. At the level of light microscopy, morphological changes are quite apparent (Figure 12, A and B). At 21 days of gestation, fetal rat liver is composed of two distinct cell components: hepatocytes and erythroid elements in roughly a one to two ratio. The hepatocyte cytoplasm is sparse and these cells seem only loosely arrayed in the sinusoidal pattern characteristic of adult (fully differentiated) liver. In contrast, the hepatocytes following 14 hours treatment with 5-azaC are much more basophilic and the sinusoidal plating of cells is better defined. The change in appearance of the liver is not due to overt hematopoietic cell cytotoxicity, as direct counts of hepatocyte and erythroid nuclei showed that both the number and ratio remained constant. Thus, qualitatively, treatment with 5-azaC caused the fetal hepatocytes to acquire a more adult morphology in utero.

To dissect the biochemical components which could one, contribute to this altered morphology and two, reflect possible enzymic or biochemical differentiation following 5-azaC treatment, a variety of additional parameters were explored. Ultrastructural analysis of fetal rat liver following 5-azaC treatment revealed significant differences in the levels of stored carbohydrate (glycogen) and lipid. The large amount of glycogen deposited in fetal hepatocytes in the days immediately prior to birth is clearly visible in 21 day fetal liver (Figure 13 A). Much of this stored glycogen is depleted following 18 hours of 5-azaC treatment, as seen in Figure 13 B. This was also apparent as the loss of PAS-positive material in

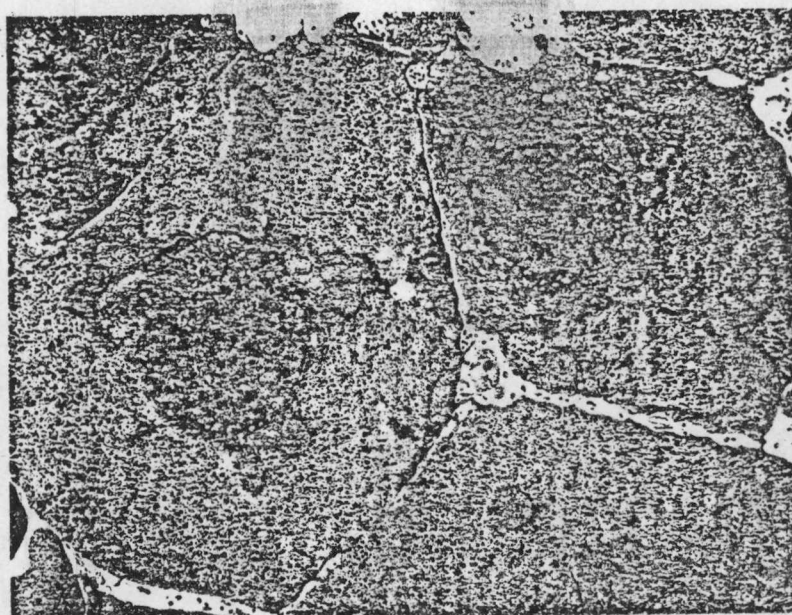


A) 460 x

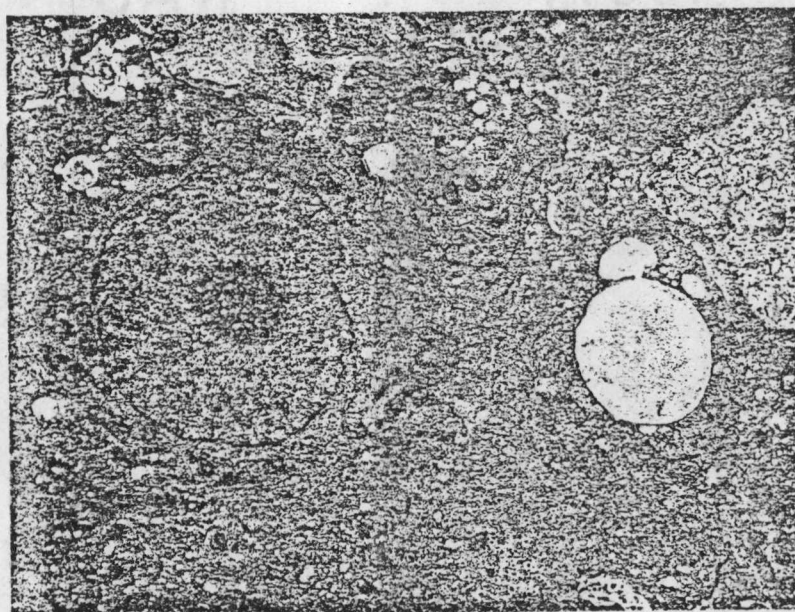


B) 460 x

Figure 12. Fetal Rat Liver Morphology. (460x). Paraffin sections of 21 day fetal rat liver (A) and 20 day fetal rat liver following 14 hours treatment with 5-azacytidine (B) were stained with hematoxylin and eosin.



A) 3400 x



B) 3400 x

Figure 13. Lipid and Glycogen Stores in Fetal Liver visualized by Electron Microscopy. (3400x). Gold sections of 21 day fetal rat liver (A) and 20 day fetal rat liver following 18 hours treatment with 5-azacytidine (B) were stained with lead citrate and uranyl acetate and low magnification electron photomicrographs taken.

histochemical staining of light microscope specimens (data not shown). Direct chemical analysis (72) of liver glycogen content showed a decrease from 61.5 ± 9.68 mg to 8.20 ± 0.58 mg per gram liver. Also striking in the ultrastructural studies are the increased lipid deposits in hepatocytes of livers from treated fetuses (Figure 13 A and B); this was also apparent in histochemical staining (not shown). Chemical analysis of lipid content revealed that total lipids increased nearly two-fold, from 13.65 mg to 24.11 mg per gram liver. Increased levels of triglycerides following treatment were found to account for the difference between the two samples when these were analyzed by thin layer chromatography. The chemical determination of lipid content for these tissues was done courtesy of Ten-Ching Lee. In some photomicrographs, the development of a smooth endoplasmic reticulum, as well as a more predominant rough endoplasmic reticulum, are evident following 5-azaC treatment.

Sucrose gradient analysis of poly(A) RNAs isolated from fetal livers following 14 hours treatment with 5-azaC revealed several interesting features relative to that of an age-matched untreated control, as seen in Figure 14. The concentration of the globin mRNA component, sedimenting at ~9S, remained constant. This is further evidence that 5-azaC does not cause erythroid cytotoxicity. There is some decrease in the amount of RNA sedimenting at 18S following treatment. The nature of that RNA sedimenting at 43S is not known, although preliminary experiments have shown it to be translationally active. This rapidly sedimenting RNA has been seen in multiple

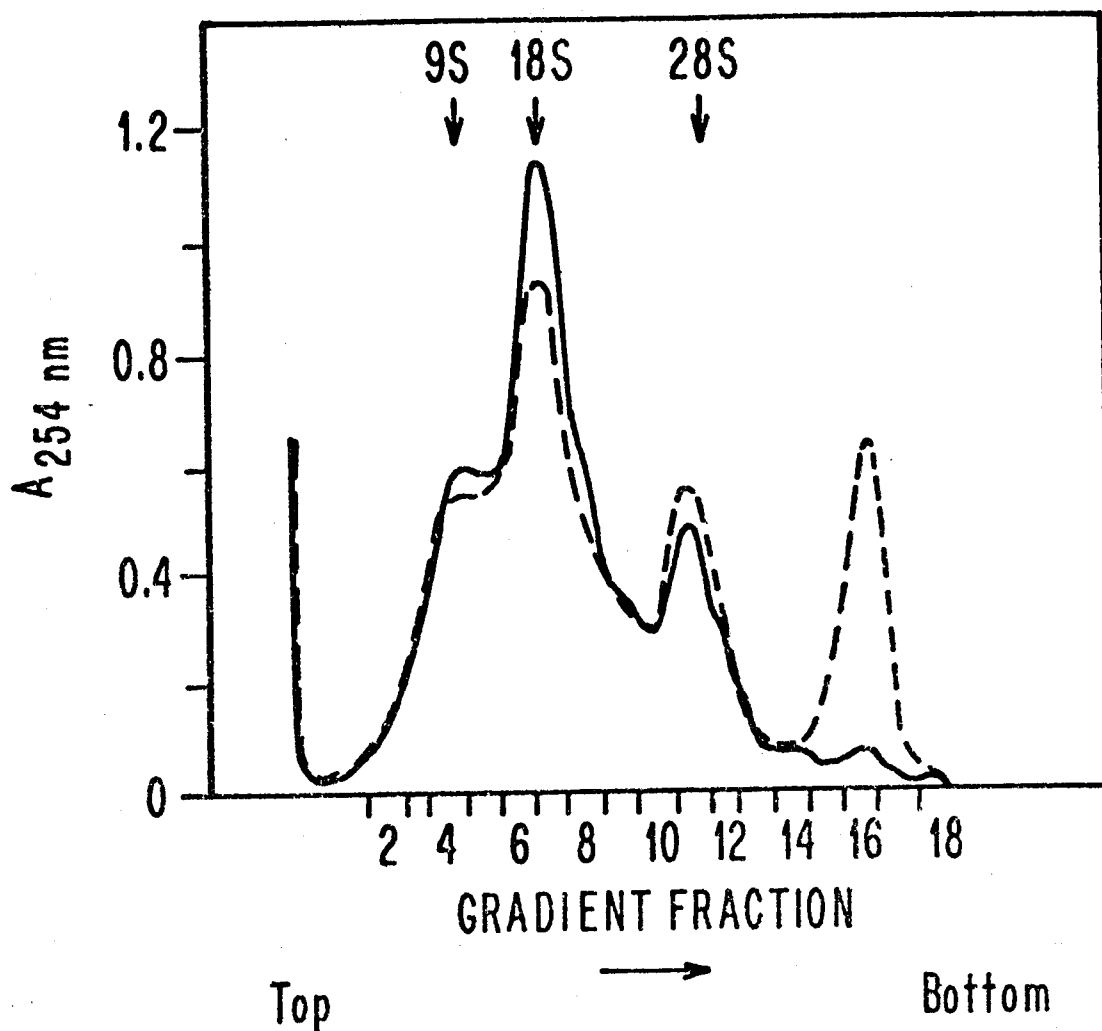


Figure 14. Sucrose Gradient Analysis of Fetal Poly(A) RNAs. Cytoplasmic poly(A) RNAs were isolated from 21 day fetal liver (solid line) and 20 day fetal liver 14 hours after treatment with 5-azacytidine (broken line) and equivalent amounts of each RNA were analyzed by centrifugation through 5 to 20% sucrose gradients. Each gradient was fractionated from top to bottom and the absorbance at 254 nm was recorded.

samples isolated following 14 hours of treatment, but was not detected in samples prepared after 9 or 18 hours of treatment.

In vitro translation products of poly(A) RNA from 21 day fetal liver and from fetal liver 18 hours after the administration of 5-azaC reveal few differences in electrophoretic profile (data not shown). Following 18 hours 5-azaC treatment, the in vitro translational capacity of the poly(A) RNA decreases to about 60% of that of the untreated fetal level.

By one-dimensional denaturing electrophoresis of 105,000 x g soluble proteins from fetal liver homogenates, and densitometric scanning of the gel, we have detected at least eight greatly increased protein bands following treatment with 5-azaC for 18 hours (Figure 15). The proteins range from 17,500 to over 100,000 daltons. Globin is missing from these gel scans as this protein is not fixed by 10% acetic acid.

We have assayed for other developmentally regulated enzymes, to see if treatment with 5-azaC activated their expression. Phosphoenolpyruvate carboxykinase is the only enzyme assayed to date whose activity increased following 18 hours treatment. The mean of two experiments showed a 10-fold induction of specific activity above fetal control levels, 0.51 ± 0.23 units to 4.98 ± 0.22 units per mg protein, but that activity was only about 25% of the steady state adult level. It is interesting that PEPCK activity should be increased, as it is in the same developmental cluster (neonatal) as TAT. Activities of glucose-6-phosphatase (which normally increases

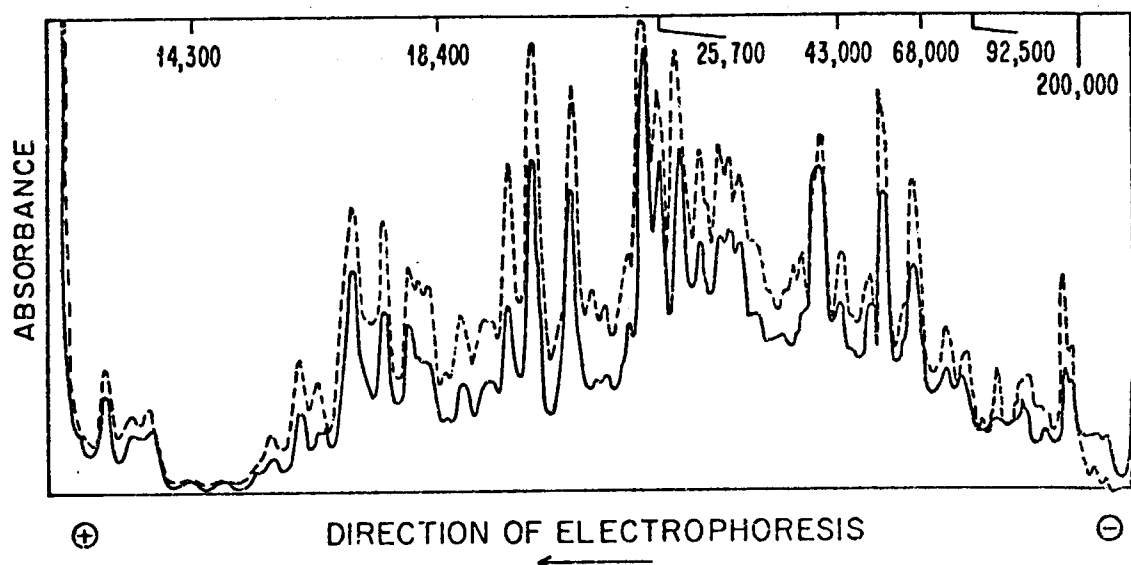


Figure 15. Electrophoretic Analysis of Soluble Proteins from Fetal Liver. 105,000 x g Supernatant fractions derived from 21 day fetal rat liver (solid line) and 21 day fetal rat liver following 18 hours of treatment with 5-azacytidine (broken line) were subjected to electrophoresis through a 10% SDS-polyacrylamide gel. Equal amounts of protein were applied for each sample. Analysis of the protein profiles was by densitometric scanning.

prior to birth) and of tryptophan dioxygenase (which appears in the weaning period) were not significantly changed by 5-azaC treatment.

We have concluded from these analyses that treatment of 20 day rat fetuses in utero with 5-azaC does exert a more extensive effect on hepatic development than activation of TAT expression alone. There are changes in the stored lipid and carbohydrate compartments, as well as in the protein and RNA populations.

Discussion

The data presented show that treatment of 20 day rat fetuses in utero with 5-azaC activates the production of TAT gene products in a pattern not unlike that seen during normal development. The action of this drug as a DNA-hypomethylating agent and the wealth of correlative evidence which suggests that key DNA-demethylations are involved in the expression of specific genes suggests that 5-azaC is activating TAT expression through this mechanism. That cytidine or analogs other than the 5-aza compound were ineffective supports this tentative conclusion. By inference, then, it seems quite likely that DNA methylation is a highly significant component of developmentally regulated expression of this gene.

I have found that response to the drug was extremely sensitive to the developmental age of the fetus; i.e., the response was greatest and most consistent when the fetuses were treated late during day 20 of gestation. The induction of fetal enzyme synthesis by 5-azaC prior to day 20 of gestation is not uniform to all littermates, nor is the level of response as high. My analysis of the appearance of TAT

gene products during normal development shows that transcripts begin to accumulate during day 21 of gestation (Figure 5, page 25), a result interpreted as indicating that the increased rate of TAT gene transcription, typical of the fully differentiated hepatocyte, begins just before this time. It is noteworthy, then, that development of what appears to be a state of "competence" enabling response to 5-azaC treatment coincides closely with the time at which transcription is normally accelerated. One could speculate that this correlation indicates that the normal developmental activation reflects demethylation, a process greatly enhanced by experimental provision of the demethylating agent. Alternatively, it may be that demethylation is necessary, but not sufficient for full activation, another change being required (and occurring during the 20 to 21 day interval) before demethylation can bring the gene to the fully activated state. The latter interpretation would be in accord with results of recent studies of developmental activation of vitellogenin synthesis in avian liver (15). Clearly an in-depth analysis of TAT gene structure and modifications during development will be required to distinguish between these (or perhaps, other) alternatives.

The question of the potential role of hormonal regulators of TAT synthesis in the activation artificially induced by 5-azaC treatment, and by inference in normal development, is a complicated one. In the fully differentiated adult liver all three types of regulators (glucocorticoids, insulin, and cAMP) modulate expression of a fully active gene (39-42), but this does not exclude the possibility that

one or more of these is required for developmental activation of gene expression. Plasma levels of glucocorticoids (38), and insulin (35), are high in late-term fetuses; either could be required for activation of the gene, or could modulate its expression once it is presumably activated by demethylation. Attempts to define a role of glucocorticoids by administration of exogenous hydrocortisone to the dam prior to analysis of 5-azaC treated fetuses were not informative, since the drug alone increased aminotransferase activity to an induced level that was not further enhanced by steroid treatment.

The activation of TAT expression following 5-azaC treatment is noteworthy in another regard, this being that the induced RNA sequences become fully functional in utero, a change of unknown nature that does not occur until after birth in normal development. One could imagine many scenarios to account for this effect, such as induced expression of a translational activator that is not normally expressed in utero, repression of an inhibitor that is normally repressed following birth, or activation of mRNA^{TAT} owing to incorporation of 5-azaC in place of cytidine and a consequent conformational change. That this drug can affect the conversion of inactive to active mRNA would appear to provide a valuable clue as to the mechanism involved, but one whose significance is not yet understood.

The effect of 5-azaC on the development of pluripotent to terminally differentiated cell lines has been documented in the literature (22,23). I have presented evidence here which suggests

that 5-azaC may exert the same effect on hepatic differentiation in vivo. This could argue for a DNA-demethylating "developmental trigger" in perinatal rat liver.

Morphological changes in the fetal liver are apparent, and are visualized by light and electron microscopy. The appearance of the tissue suggests a more mature, differentiated hepatocyte population.

The glycogen stores deposited in fetal hepatocytes during the last days of gestation are prominent in electron photomicrographs. By this same analysis, hepatocytes following 5-azaC treatment show a diminution of stored glycogen and an increase in intracellular lipid. The biochemical rationale for this metabolic shift is not known. Several hypotheses can be put forth. The depletion of glycogen could be in response to increased energy demands that post-partum are met by the release of stored glucose into the circulatory system. However, in utero, the glucose carbons may instead become precursor to lipogenesis. The increase in stored lipid could also reflect decreased lipid transport out of the liver, known to be an element of the "fatty liver" response to toxic agents.

Translation of poly(A) RNA and electrophoretic analysis of those products suggested that globin synthesis may be slightly reduced while the synthesis of some non-globin proteins may be increased following 5-azaC treatment. However, interpretation of these protein profiles is complicated by the reduced translational capacity of the poly(A) mRNA population isolated following 14 or 18 hours treatment with 5-azaC. Densitometric analyses of soluble protein samples also suggest that there is more non-globin protein in the homogenates

derived from 5-azaC treated fetuses. However, superimposed on this general increase in the non-globin protein component, several proteins are increased dramatically, including those migrating at approximately 17,250, 21,750, and 30,300 daltons (Figure 15).

Interpretation of the activity measurements of other developmentally regulated liver enzymes is equivocal at best. The assay of enzyme activity as evidence of specific gene activation is limited by the activity of its cognate mRNA, which could well be rendered inactive or partially active by incorporation of 5-azaC. Levitan and Webb have presented data on effects of this drug in adult liver that could be interpreted as a selective inhibition of mRNA activity owing to 5-azaC incorporation (73). Also, the response of a given gene is likely to be dependent on the sequence of other developmental events and/or competencies.

In conclusion, treatment of rat fetuses two days prior to normal delivery elicited a dramatic change in fetal liver morphology and metabolism. Hepatic maturation and differentiation appear to have been accelerated. The response of the developmentally regulated enzyme, TAT, was best described; the appearance and activity of the gene products were found to closely mimic the pattern of normal development seen in neonates.

CHAPTER IV

SUMMARY

Earlier studies of the developmental activation of TAT expression had led to the concept that expression is essentially repressed throughout gestation, release from the intrauterine environment somehow triggering production of gene products. The present data do not contradict this, but establish that the activation process is more complex and more interesting than had been previously anticipated. Accelerated transcription of the gene begins the day prior to birth and has reached adult or greater than adult levels of activity in the newborn. This is indicated by the accumulation of both cytoplasmic and nuclear transcripts. Superimposed on this transcriptional control is a post-transcriptional mechanism acting to limit the activity of those transcripts until several hours after birth. Newly synthesized fetal or newborn transcripts do not function either in the hepatocyte or in the heterologous translation system.

In utero enzyme synthesis is refractory to activation by the hormones which modulate TAT expression in the adult (39-42, 44-46). Further, the postnatal surge in functional gene products (from fetal to adult levels) occurs independently of steroid induction (44, 47). Thus, it was inferred that these modulators play an insignificant role in the course of developmental activation. This view will have to be revised in light of very recent results in our laboratory (A.C. Johnson, research in progress). These data clearly establish that TAT expression in utero is not entirely refractory to induction by

steroids. Hybridizable transcripts increase markedly after direct treatment of fetuses with hydrocortisone. But as in the untreated newborn, the induced transcripts remain non-functional; there is no change detectable in mRNA^{TAT} or enzyme activities. These results support the concept that a post-transcriptional block, preventing translation of aminotransferase mRNA, is operating throughout fetal life.

Elucidation of the molecular details involved in both transcriptional and post-transcriptional controls which operate in TAT gene activation can be expected to be greatly facilitated by the finding that expression can be fully activated, in utero, following administration of 5-azaC. Perhaps even more remarkable are the indications that hepatocyte maturation, and not just the expression of this single gene, is markedly accelerated by this inhibitor of DNA methylation. Changes in the liver content of RNA and of proteins, of at least one specific enzyme other than TAT, and of glycogen and of lipids are all suggestive of a significant role of gene methylation in many aspects of hepatic differentiation. Further work is required to delineate fully the biochemical changes responsible for the change in hepatocyte morphology evident here; to distinguish between those changes induced by 5-azaC acting as an inhibitor of DNA methylation and its other potential biological effects.

The primary effect of this drug on TAT expression is in accord with its role as a DNA demethylating agent: transcription of this gene is apparently accelerated after treatment with 5-azaC. Hybridizable cytoplasmic sequences are increased after six hours of

treatment and at all time points assayed thereafter are well above adult levels. This result strongly suggests that DNA methylation plays a dominant role in limiting TAT transcription prior to birth. To pursue this further requires the isolation and characterization of the TAT gene in search of those key regulatory sequences that may be affected by 5-azaC treatment. Isolation of this gene from a clone library of rat genomic DNA is currently in progress in our laboratory.

The sequence of appearance of the TAT gene products following 5-azaC treatment is remarkably similar to the pattern seen in normal development, especially in the lag between the accumulation of hybridizable cytoplasmic sequences and their acquisition of functional capacity. Noteworthy then is the observation that the sequences do become functional in utero. This indicates that treatment with 5-azaC not only accelerates TAT transcription, but also overcomes the post-transcriptional block which limits full expression of the gene at this time of development. The mechanism of this is not now understood, but it is likely that this finding will be useful in defining the changes involved in the transition from inactive to active mRNA^{TAT}.

The picture of TAT development activation arising from these studies is one of a highly ordered, temporally programmed series of molecular events acting sequentially and culminating in the postnatal production of the final gene product, the enzyme. Accelerated transcription begins the day prior to birth, perhaps following demethylation of one or more regulatory sites in the DNA. The inconsistency of TAT expression in response to 5-azaC treatment prior to day 20 implies that an earlier change of some sort is required; if so,

this event is somewhat analogous to the "commitment" phenomenon which precedes the expression of a differentiated phenotype. (For a discussion of these concepts, see the reviews by Dawid and Wahli [74], and by Paigen [75].) Following TAT transcriptional activation, there is a period wherein post-transcriptional control operates to limit full expression; after birth (or following treatment with 5-azaC) this control is lifted and enzyme synthesis begins.

LIST OF REFERENCES

LIST OF REFERENCES

1. Holliday, R., and J.E. Pugh. (1975). DNA modification mechanisms and gene activity during development. *Science* 187, 226-232.
2. Sager, R., and R. Kitchin. (1979). Selective silencing of eukaryotic DNA. *Science* 189, 426-433.
3. Razin, A., and A.D. Riggs. (1980). DNA methylation and gene function. *Science* 210, 604-610.
4. Lindahl, T. (1981). DNA methylation and control of gene expression. *Nature* 290, 363-364.
5. Ehrlich, M., and R.Y.-H. Wang. (1981). 5-Methylcytosine in eukaryotic DNA. *Science* 212, 1350-1357.
6. Felsenfeld, G., and J. McGhee. (1982). Methylation and gene control. *Nature* 296, 602-603.
7. Doerfler, W. (1983). DNA Methylation and Gene Activity. In: *Ann. Rev. Biochem., Snell, E.E., P.D. Boyer, A. Meister, and C.C. Richardson (eds.) Ann. Reviews Inc., pp. 93-124.*
8. Naveh-Manly, T., and H. Cedar. (1981). Active gene sequences are undermethylated. *Proc. Natl. Acad. Sci. USA* 78, 4246-4250.
9. Bird, A.P., H.H. Taggart, and B.A. Smith. (1979). Methylated and unmethylated DNA compartments in the sea urchin genome. *Cell* 17, 889-901.
10. van der Ploeg, L.H.T., and R.A. Flavell. (1980). DNA methylation in the human $\gamma\delta\beta$ -globin locus in erythroid and nonerythroid tissues. *Cell* 19, 947-958.
11. Mandel, J.L., and P. Chambon. (1979). DNA methylation: organ specific variations in the methylation pattern within and around ovalbumin and other chicken genes. *Nucl. Acids Res.* 7, 2081-2103.
12. Kuo, M.T., J.L. Mandel, and P. Chambon. (1979). DNA methylation: correlation with DNase I sensitivity of chicken ovalbumin and conalbumin chromatin. *Nucl. Acids Res.* 7, 2105-2113.
13. Stein, R., N. Sciaky-Gallili, A Razin, and H. Cedar. (1983). Pattern of methylation of two genes coding for housekeeping functions. *Proc. Natl. Acad. Sci. USA* 80, 2422-2426.

14. Weintraub, H., A. Larsen, and M. Groudine. (1981). α -Globin-gene switching during the development of chick embryos: expression and chromosome structure. *Cell* 24, 333-344.
15. Burch, J.B.E., and H. Weintraub. (1983). Temporal order of chromatin structural changes associated with activation of the major chick vitellogenin gene. *Cell* 33, 65-76.
16. Wilks, A.F., P.J. Cozens, I.W. Mattaj, and J.-P. Jost. (1982). Estrogen induces a demethylation at the 5' end region of the chick vitellogenin gene. *Proc. Natl. Acad. Sci. USA* 79, 4252-4255.
17. Rogers, J., and R. Wall. (1981). Immunoglobulin heavy chain genes: demethylation accompanies class switching. *Proc. Natl. Acad. Sci. USA* 78, 7497-7501.
18. Creusot, F., G. Acs, and J.K. Christman. (1982). Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'-deoxycytidine. *J. Biol. Chem.* 257, 2041-2048.
19. Gjerset, R.A., and D.W. Martin. (1982). Presence of a DNA demethylating activity in the nucleus of murine erythroleukemic cells. *J. Biol. Chem.* 257, 8581-8583.
20. Cihák, A. (1974). Biological effects of 5-azacytidine in eukaryotes. *Oncology* 30, 405-422.
21. Mohandas, T., R.S. Sparkes, and L.J. Shapiro. (1981). Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. *Science* 211, 393-396.
22. Taylor, S.M., and Jones, P.A. (1979). Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell* 17, 771-779.
23. Jones, P.A., and S.M. Taylor. (1980). Cellular differentiation, cytidine analogs, and DNA methylation. *Cell* 20, 85-93.
24. Sager, R., and P. Kovac. (1982). Pre-adipocyte determination either by insulin or by 5-azacytidine. *Proc. Natl. Acad. Sci. USA* 79, 480-484.
25. Groudine, M., R. Eisenman, and H. Weintraub. (1981). Chromatin structure of endogenous retroviral genes and activation by an inhibitor of DNA methylation. *Nature* 292, 311-317.

26. Compere, S.J., and R.D. Palmiter. (1981). DNA methylation controls the inducibility of the mouse metallothionein-I gene in lymphoid cells. *Cell* 25, 233-240.
27. Ley, T.J., J. DeSimone, N.P. Anagnou, G.H. Keller, R.K. Humphries, P.H. Turner, N.S. Young, P. Heller, and A.W. Nienhuis. (1982). 5-Azacytidine selectively increases γ -globin synthesis in a patient with β^+ thalassemia. *N. Eng. J. Med.* 307, 1469-1475.
28. Santi, D.V., C.E. Garrett, and P.J. Barr. (1983). On the mechanism of inhibition of DNA-cytosine methyltransferases by cytosine analogs. *Cell* 33, 9-10.
29. Greengard, O., M. Federman, and W.E. Knox. (1972). Cyto-morphology of developing rat liver and its application to enzymic differentiation. *J. Cell Biol.* 52, 261-272.
30. Oliver, I.T., F.J. Ballard, J. Shield, and P.J. Bentley, (1962). Liver growth in the early postpartum rat. *Dev. Biol.* 4, 108-116.
31. Oliver, I.T., W.F.C. Blumer, and I.J. Witham. (1963). Free ribosomes during maturation of rat liver. *Comp. Biochem. Physiol.* 10, 33-38.
32. Greengard, O. (1971). Enzymic differentiation in mammalian tissues. In: *Essays in Biochemistry* 7, Campbell, P.N., and F. Dickens (eds) Academic Press, pp. 159-205.
33. Barth, R.K., K.W. Gross, L.C. Gremke, and N.D. Hastie. (1982). Developmentally regulated mRNAs in mouse liver. *Proc. Natl. Acad. Sci. USA* 79, 500-504.
34. Greengard, O. (1969). Enzymic differentiation in mammalian liver. *Science* 163, 891-895.
35. Girard, J.R., G.S. Cuendet, E.B. Marliss, A. Kervran, M. Rieutort, and R. Assan. (1973). Fuels, hormones, and liver metabolism at term and during the early postnatal period in the rat. *J. Clin. Invest.* 52, 3190-3200.
36. Walker, D.G. (1971). Development of enzymes for carbohydrate metabolism. In: *The Biochemistry of Development*, Benson, P. (ed.) J.B. Lippincott Co., pp. 77-95.
37. Greengard, O. (1975). Steroids and the maturation of rat tissues. *J. Steroid Biochem.* 6, 639-642.

38. Holt, P.G., and I.T. Oliver. (1968). Plasma corticosterone concentrations in the perinatal rat. *Biochemistry J.* 108, 339-341.
39. Lin, E.C.C., and W.E. Knox. (1957). Adaptation of the rat liver tyrosine- α -ketoglutarate transaminase. *Biochem. Biophys. Acta* 26, 85-88.
40. Kenney, F.T. (1962). Induction of tyrosine- α -ketoglutarate transaminase in rat liver: evidence for an increase in the rate of enzyme synthesis. *J. Biol. Chem.* 237, 3495-3499.
41. Holten, D., and F.T. Kenney. (1967). Regulation of tyrosine α -ketoglutarate transaminase in rat liver: induction by pancreatic hormones. *J. Biol. Chem.* 242, 4372-4377.
42. Wicks, W.D., F.T. Kenney, and K.-L. Lee. (1969). Induction of hepatic enzyme synthesis in vivo by adenosine 3',5'-monophosphate. *J. Biol. Chem.* 244, 6008-6013.
43. Stiles, C.D., K.-L. Lee, and F.T. Kenney. (1976). Differential degradation of messenger RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA* 73, 2634-2638.
44. Sereni, F., F.T. Kenney, and N. Kretchmer. (1959). Factors influencing the development of tyrosine- α -ketoglutarate transaminase activity in rat liver. *J. Biol. Chem.* 234, 609-612.
45. Greengard, O., and H.K. Dewey. (1967). Initiation by glucagon of the premature development of tyrosine aminotransferase, serine dehydratase and glucose-6-phosphatase. *J. Biol. Chem.*, 242, 2986-2991.
46. Adelman, R.C. (1971). A possible role for pituitary function in the age dependent regulation of hepatic tyrosine aminotransferase activity. *Biochem. Biophys. Res. Commun.* 43, 814-818.
47. Perry, S.T., R. Rothrock, K.R. Isham, K.-L. Lee, and F.T. Kenney. (1983). Development of tyrosine aminotransferase in perinatal rat liver: changes in functional messenger RNA and the role of inducing hormones. *J. Cell Biochem.* 21, 47-61.
48. Wicks, W.D. (1968). Induction of tyrosine α -ketoglutarate transaminase in fetal rat liver. *J. Biol. Chem.* 243, 900-906.
49. Geoghegan, G., S. Cereghini, and G. Brawerman. (1979). Inactive mRNA-protein complexes from mouse sarcoma-180 ascites cells. *Proc. Natl. Acad. Sci. USA* 76, 5587-5591.

50. Yenofsky, R., I. Bergmann, and G. Brawerman. (1982). Messenger RNA species partially in a repressed state in mouse sarcoma ascites cells. *Proc. Natl. Acad. Sci. USA* 79, 5876-5880.
51. Palmiter, R.D. (1974). Magnesium precipitation of ribonucleo-protein complexes. Expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. *Biochemistry* 13, 3606-3615.
52. Krystosek, A., M.L. Cawthon, and D. Kabat. (1975). Improved methods for purification and assay of eukaryotic messenger ribonucleic acids and ribosomes. *J. Biol. Chem.* 250, 6077-6084.
53. Pelham, H.R.B., and R.J. Jackson. (1976). An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67, 247-256.
54. Mans, R.J., and G.D. Novelli. (1961). Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. *Arch. Biochem. Biophys.* 94, 48-53.
55. Weber, K. and M. Osborn. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244, 4406-4412.
56. Nickol, J.M., K.-L. Lee, T.G. Hollinger, and F.T. Kenney. (1976). Translation of messenger RNA specific for tyrosine aminotransferase in oocytes of *Xenopus laevis*. *Biochem. Biophys. Res. Commun.* 72, 687-698.
57. Spelsberg, T.C., J.T. Knowler, and H.L. Moses. (1974). Specific methods for the isolation of nuclei from chick oviduct. In: *Methods Enzymol.* 31, Fleischer, S., and L. Packer (eds.) Academic Press, pp. 263-279.
58. Busch, H., and K. Smetana. (1970). Isolation and purification of nuclei and nucleoli. In: *The Nucleolus*. Academic Press, pp. 512-531.
59. Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18, 5294-5299.
60. Thomas, P.S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* 77, 5201-5205.

61. O'Farrell, P.H., E. Kutter, and M. Nakanishi. (1980). A restriction map of the bacteriophage T4 genome. *Molec. Gen. Genet.* 179, 421-435.
62. White, B.A., and F.C. Bancroft. (1982). Cytoplasmic dot hybridization. Simple analysis of relative mRNA levels in multiple small cell or tissue samples. *J. Biol. Chem.* 257, 8569-8572.
63. Ruiz-Bravo, N., and M.J. Ernest. (1982). Induction of tyrosine aminotransferase mRNA by glucocorticoids and cAMP in fetal rat liver. *Proc. Natl. Acad. Sci. USA* 79, 365-368.
64. Benyajati, C., N. Spoerel, H. Haymerle, and M. Ashburner. (1983). The messenger RNA for alcohol dehydrogenase in Drosophila melanogaster differs in its 5' end in different developmental stages. *Cell* 33, 125-133.
65. Merlino, G.T., C. McKeon, B. deCrombrughe, and I. Pastan. (1983). Regulation of the expression of genes encoding types I, II and III collagen during chick embryonic development. *J. Biol. Chem.* 258, 10041-10048.
66. Arnstein, H.R.V. (1982). Messenger ribonucleoprotein complexes in gene expression. In: *Biochem. Soc. Symp.* 47. Phelps, C.F., and H.R.V. Arnstein (eds.). *Biochem. Soc.*, pp. 163-182.
67. Kenney, F.T. (1959). Properties of partially purified tyrosine- α -ketoglutarate transaminase from rat liver. *J. Biol. Chem.* 234, 2707-2712.
68. Ballard, F.J. and R.W. Hanson. (1967). Phosphoenolpyruvate carboxykinase and pyruvate carboxylase in developing rat liver. *Biochem. J.* 104, 866-871.
69. Burch, H.B., O.H. Lowry, A.M. Kuhlman, J. Skerjance, E.J. Diamant, S.R. Lowry, and P. VonDippe. (1963). Changes in patterns of enzymes of carbohydrate metabolism in the developing rat liver. *J. Biol. Chem.* 238, 2267-2273.
70. Schutz, G., and P. Feigelson. (1972). Purification and properties of rat liver tryptophan oxygenase. *J. Biol. Chem.* 247, 5327-5332.
71. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

72. Hassid, W.Z., and S. Abraham. (1957). Chemical procedures for analysis of polysaccharides. In: *Methods Enzymol.* 3, Colowick, S.P., and N.O. Kaplan (eds.) Academic Press, pp. 34-50.
73. Levitan, I.B., and T.E. Webb. (1969). Effects of 5-azacytidine on polyribosomes and on the control of tyrosine transaminase activity in rat liver. *Biochem. Biophys. Acta* 182, 491-500.
74. Dawid, I.B., and W. Wahli. (1979). Application of recombinant DNA technology to questions of developmental biology: a review. *Dev. Biol.* 69, 305-328.
75. Paigen, K. (1979). Genetic factors in developmental regulation. In: *Physiological Genetics*, Scandalios, J.D. (ed.) Academic Press, pp. 1-61.

VITA

Robin Stephanie Rothrock was born 6 June 1956 in Washington, DC. She graduated first in her class from Loudoun County High School, Leesburg, Virginia, in June 1974. She obtained her A.B. from Hollins College, Hollins College, Virginia in May 1978, graduating with honors, departmental honors in biology, and membership in Phi Beta Kappa (February 1977) and Sigma Xi. She began her graduate career at The University of Tennessee, Knoxville in September 1978 and was awarded the degree of Doctor of Philosophy in Biomedical Sciences in December 1983.