Nuclear Ribonucleic Acid in Embryonic Rat Liver*

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ABSTRACT: The biosynthesis and properties of nuclear ribonucleic acid (nRNA) of rat fetal liver were examined. Sedimentation analysis of fetal liver nRNA revealed the presence of four peaks of ultraviolet absorption, at 4–6, 18, 28, and 45 S; a shoulder was noted in the 35S region. After an *in vivo* pulse of 1 hr with [^{14}C]orotic acid, the nRNA with the highest specific activity was recorded in the region between 6 and 18, 18 and 28, and at 45 S. The base compositions of the 4–6S, 18S, and 28S fractions of fetal liver nRNA were determined. The per cent guanosine monophosphate (GMP) plus cytidine monophosphate (CMP) was 50.0, 55.8, and 63.7%, respectively. The messenger-like activity of fetal liver nRNA and of its components was ascertained in an *Escherichia coli* protein-synthesizing system. Fetal and adult liver nRNA both stimulated the incorporation of [^{14}C]valine into polypeptide material. The stimulatory influence was blocked by puromycin, streptomycin, chloramphenicol, and RNase; cycloheximide was without effect. Neomycin in amounts less than 1 μg increased the template activity of both boiled and unboiled fetal liver nRNA preparations while above this amount, an inhibition was apparent. The greatest template activity was observed in the 18S and 45S regions of the sucrose density gradient.

The development of mammalian liver from the embryonic to the adult stage is accompanied by a profound alteration in function. While hematopoiesis is the essential function of early fetal liver, it plays only a secondary role in the intracellular activities of the adult hepatocyte. On the other hand, the transformation of foreign agents and of naturally occurring steroids, is almost undetectable in fetal liver while adult liver conducts these processes quite efficiently (Fouts and Adamson, 1959; Fouts and Hart, 1965; Kuntzman *et al.*, 1965).

In accordance with the current status of our knowledge, these changes in genetic expression should be accompanied by alterations in mRNA profiles. A study has been initiated in this laboratory with the ultimate aim of characterizing the phases of differentiation of liver in terms of its nuclear ribonucleic acid (nRNA) complement. The initial obstacle to this study was the adaptation of the existing methodology to the purification of sufficient quantities of RNA from preparations of fetal liver. This test has been met and the results have been presented in an earlier publication (Bresnick *et al.*, 1967). The present study was devoted to an analysis of the fetal liver nRNA and especially to its template, *i.e.*, messenger-like, activity as determined in a cell-free *Escherichia coli* amino acid incorporating system. The results of this study are offered in this manuscript.

Materials and Methods

Pregnant rats (18–20 days of gestation) were purchased from the Cheek-Jones Co., Houston, Texas, and were allowed free access to both food and water.

Preparation of Nuclei. The adult rats were exsanguinated by transection of the renal artery and vein and the livers were perfused *via* the portal system *in situ* with cold 0.25 M sucrose. The perfused livers were removed, blotted, and weighed. The remaining procedures were conducted in a cold room at 5°. The perfused livers were minced in a Harvard tissue mincer and the mince was homogenized in 2.4 M sucrose–3 mM $CaCl_2$ (1:12, w/v) (Chauveau *et al.*, 1956). The homogenate was centrifuged at 40,000*g* for 60 min to yield a crude nuclear pellet which was rehomogenized in 1 M sucrose–1 mM $CaCl_2$ (1 ml/g of liver) and centrifuged at 900*g* for 10 min. The purified nuclear pellet was then utilized in the preparation of nRNA.

Fetal liver nuclei were isolated by the procedure recently described (Bresnick *et al.*, 1967). In this procedure, the fetal liver was washed in cold 0.25 M sucrose, homogenized in 2.4 M sucrose–3 mM $CaCl_2$ (1:15, w/v), and the molarity of the sucrose in the homogenate was then adjusted to 2.3 M. The homogenate was centrifuged at 40,000*g* for 60 min to yield the crude red nuclear pellet. The latter was purified by suspension in 1 M sucrose–1 mM $CaCl_2$. The sedimented pellet was suspended in 0.3 mM phosphate buffer (pH 6.8)–0.3 mM $MgCl_2$ and layered over an equal volume of 1 M sucrose–1 mM $CaCl_2$. The purified nuclear pellet was obtained after centrifugation at 1000*g* for 10 min.

The purity of the nuclear preparations was assessed

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by phase and light microscopy. For the latter, the nuclei were stained with a solution of 0.05% azure C in 0.25 M sucrose.

Preparation of Labeled Fetal Liver nRNA. The RNA was labeled *in vivo* as described previously (Bresnick *et al.*, 1965). Pregnant Cheek-Jones rats were placed under ether anesthesia, an abdominal incision was made, and the uterine horns containing the fetuses were exposed. [6-^{14}C]Orotic acid (0.2 μc, 3.5–5.0 μc/μmole) was delivered into each amniotic sac by means of a 30-gauge needle attached to a 0.25-ml syringe. The uterine horns were replaced in the abdominal cavity. Periodically, the fetuses were removed from the sacs and decapitated. The fetal livers were extirpated, washed in cold 0.25 M sucrose, and placed in a weighed beaker in an ice bath. The fetal livers from five to ten pregnant rats (*i.e.*, 40–100 fetuses) were combined and used in the isolation of the labeled nRNA by the method of Steele *et al.* (1965).

The supernatant remaining after the sedimentation of the crude nuclear pellet was diluted tenfold with distilled water and the cytoplasmic ribonucleic acid (cRNA) was extracted by the phenol method. The concentration of the RNA was determined by the orcinol procedure (Drury, 1948).

Preparation of RNA. RNA was isolated from a 100,000*g* supernatant preparation of rat liver by the phenol method (Steele *et al.*, 1965). The RNA components were extracted from cRNA by an overnight treatment with 1 M NaCl. The RNA was precipitated from the 1 M NaCl extract by two volumes of 95% ethanol–2% potassium acetate.

Sedimentation Analysis. An aliquot of the nRNA solution (0.1–0.4 ml), containing approximately 10 A_{260} units, was layered over 26.5 ml of a 10–40% linear sucrose gradient which was buffered to pH 5.1 with 0.01 M sodium acetate and also contained 0.1 M NaCl and 1 mM EDTA (Scherrer and Darnell, 1962). The tubes were then centrifuged at 24,000 rpm in a Spinco SW 25.1 swinging-bucket rotor for 15 hr at 5°. The gradients were fractionated into 1-ml samples by means of an Isco automatic fractionator which recorded the A_{254} automatically. The samples were either prepared for counting or assayed for template activity as described below. In the former, 2 drops of perchloric acid was added to the RNA sample and the mixture was incubated at 70° for 30 min. Bray's (1960) phosphor (8 ml) was then added and the samples were counted in a Packard liquid scintillation spectrometer with an efficiency of 70%.

Base Composition. Fractions from the sucrose density gradients were combined to yield the 4–6S, 18S, and 28S RNA. The RNA fractions were dialyzed against sterile distilled water overnight. To each fraction was added 95% ethanol and sufficient potassium acetate to yield a 2% solution. The suspension was kept at −10° overnight and then the RNA was sedimented by centrifugation. To each fraction 1 ml of 0.3 N KOH was added and the RNA was hydrolyzed at 80° for 45 min. The solution was neutralized with 6 N perchloric acid at 4° and the $KClO_4$ precipitate was removed by centrifugation. To 0.5 ml of the solution containing the RNA nucleotides was added 0.5 ml of 0.1 N HCl and the sample was then applied to a 0.9 × 5.0 cm column of Dowex 50-X4 (H^+, 200–400 mesh) that had previously been washed with 3 N HCl, water until neutral, and equilibrated with 20 ml of 0.05 N HCl. The ribonucleotides were then separated by the method of Katz and Comb (1963). The quantity of the nucleotide was estimated by employing the following extinction coefficients (M^{-1} cm^{-1}): UMP,[1] E_{260} 10,000; GMP, E_{257} 12,000; CMP, E_{279} 13,000; and AMP, E_{257} 14,900 (Hirsch, 1966).

Determination of Messenger-Like Activity. In the initial studies, *E. coli* B was routinely grown at 37° with aeration in a salts–glucose medium. A more active amino acid incorporating system, however, was obtained by employing a step-up culture method originally suggested to us by Drs. T. F. Dunn and A. C. Griffin of the M. D. Anderson Hospital and Tumor Institute. In this method, an inoculum of *E. coli* B is grown at 37° for 8–12 hr in 45 ml of a medium consisting of $(NH_4)_2SO_4$ (3 g/l.), K_2HPO_4 (7.4 g/l.), KH_2PO_4 (3 g/l.), NaCl (0.1 g/l.), $MgSO_4$ (0.1 g/l.), and glucose (5 g/l.). The inoculum was then added to 5 l. of a medium of the same composition except glucose was present at only 1.25 g/l. The organism was incubated at 37° overnight with aeration. The growth of the organism was then stepped-up by the further addition of 5 l. of culture medium and the adjustment of the glucose concentration to 5 g/l. The stepped-up culture was incubated again at 37° with vigorous aeration and the A_{630} was monitored at 15-min intervals in a Bausch and Lomb spectrophotometer. The *E. coli* was harvested by means of a Sharples centrifuge when the A_{630} reached 0.60–0.65. The increment in A_{630} under the conditions outlined above was generally 0.2 A/hr.

The S-30 fraction was prepared, treated with DNase, and preincubated as described by Nirenberg and Matthaei (1961). The only exception was the incorporation of NH_4^+ in place of some of the K^+ (see below) (Conway, 1964). The protein concentration of the extract was determined by the Lowry method (Lowry *et al.*, 1951). The *E. coli* cell-free system for incorporating amino acids included in a volume of 0.25 ml: DNase-treated, preincubated S-30 extract, 1–2 mg of protein; Tris buffer (pH 7.8), 25 μmoles; β-mercaptoethanol, 1.0 μmole; ATP, 0.25 μmole; GTP, 7.5 mμmoles; magnesium acetate, 3 μmoles; NH_4Cl, 12.5 μmoles; KCl, 3 μmoles; PEP, 1.9 μmoles; pyruvate kinase, 4.0 μg; *E. coli* RNA, 100 μg; 20 amino acids minus the labeled one, 0.05 μmole; L-[μ-^{14}C]valine (208.5 mc/mmole), 0.25 μc or L-[μ-^{14}C]phenylalanine (16.7 mc/mmole), 0.5 μc; poly U, nRNA, or fractions from

[1] Abbreviations used: UMP, uridine monophosphate; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; PEP, phosphoenolpyruvate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; AMP, adenosine monophosphate; TCA, trichloroacetic acid; TMP, thymidine monophosphate.

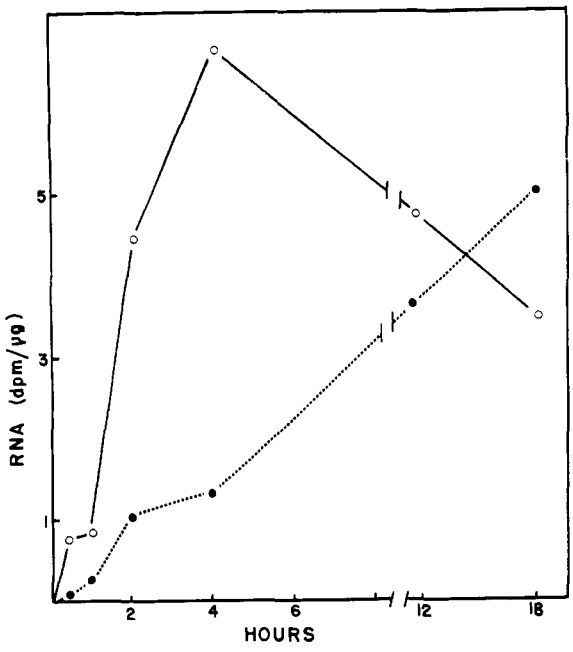

FIGURE 1: Kinetics of the *in vivo* synthesis of RNA in fetal liver. See text for details. Each point represents the average of three determinations (each determination was made upon the nRNA from the fetuses of five rats). (O——O) nRNA. (●——●) cRNA.

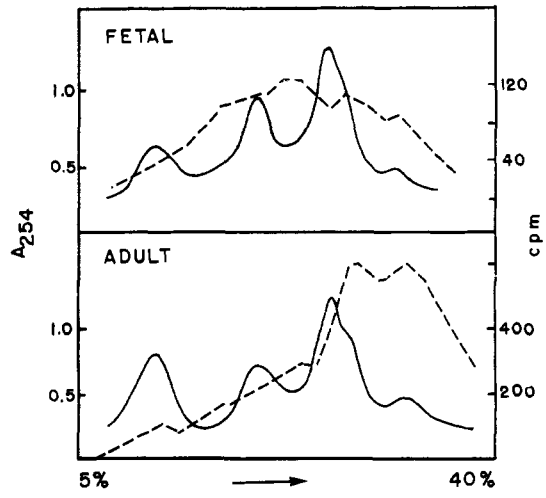

FIGURE 2: Sedimentation analysis of liver nRNA. See the text for some of the experimental details. Approximately 20 A_{254} units of nRNA from fetal liver (top) or adult liver (bottom) were applied to 5–40% linear sucrose to gradients. The adult liver nRNA had been labeled after the intraperitoneal administration of [6-^{14}C]orotic acid (5 μc/μmole, 2 μc) for 1 hr. The fetal liver nRNA was labeled as described in the text. The top of the gradient, *i.e.*, 5%, is represented at the left of the figure. The direction of sedimentation is from left to right (arrow). The peaks of absorbance (solid line) correspond to 4–6, 18, 28, and 45 S with a shoulder at 35 S. The dashed lines indicate the radioactivity.

the sucrose density gradient. The fetal liver nRNA was dialyzed against sterile distilled water for 2 hr in a rocking dialyzer to remove any traces of ethanol. The incubation was conducted for 15 min at 37°. The test tubes were immersed in an ice bath and 50-μl aliquots were withdrawn and placed on disks, 2 cm in diameter, of Whatman 3MM paper (Griffin *et al.*, 1965). The disks were immersed in cold 5% TCA, removed, and washed twice in additional cold 5% TCA, heated 15 min in 5% TCA at 90°, and washed twice with cold 95% ethanol. The disks were dried at 80° and counted in a toluenephosphor in a Packard Tri-Carb scintillation spectrometer with a counting efficiency of 50%.

Materials. The *E. coli* RNA, poly U, pyruvate kinase, PEP, ATP, GTP, and amino acids were purchased from Calbiochem. Puromycin, streptomycin, and cycloheximide were purchased from the Nutritional Biochemical Corp. Neomycin was a gift from E. R. Squibb while chloramphenicol was obtained from Dr. M. Lane of this Department. The ^{14}C-labeled amino acids and [^{14}C]orotic acid were obtained from New England Nuclear Corp. The original slant of *E. coli* was kindly donated by Drs. A. C. Griffin and T. F. Dunn of the M. D. Anderson Hospital and Tumor Institute.

Results

The kinetics of the *in vivo* incorporation of [^{14}C]-orotic acid into nuclear and cRNA of fetal rat liver are indicated in Figure 1. The nRNA was rapidly labeled reaching a maximum of 7 dpm/μg at 4 hr after administration of the isotope. By 18 hr, the specific activity was 3.5 dpm/μg of RNA. cRNA, on the other hand, was very slowly labeled; the maximum value, in this study, was not yet reached at 18 hr after administration of the labeled precursor.

The fetal and adult liver nRNA were subjected to sedimentation analysis on sucrose density gradients. Fetal liver nRNA exhibited peaks of ultraviolet absorption at 4–6, 18, 28, and 45 S; a shoulder was always observed in the 35S region. The distribution of radioactivity was recorded after an *in vivo* pulse for 1 hr with [^{14}C]orotic acid (Figure 2). The RNA with the highest specific activity, approximately 200 cpm/A_{254}, was observed in the region between 6 and 18, 18 and 28, and at 45 S. These data suggest the simultaneous synthesis of these RNA components or the catabolism of an RNA molecule, perhaps of the 45S variety, to the other substances.

Sedimentation analysis of adult liver nRNA yielded similar results presented in the bottom half of Figure 2. The sedimentation patterns, although qualitatively similar to the fetal liver nRNA indicated a larger amount of 4–6S RNA and a sharper definition of 35S and 45S components. After a 1-hr pulse with orotic

TABLE I: Base Composition of Components of Fetal Liver nRNA.[c]

Fraction	Mole %				% GMP + CMP	GMP + CMP:AMP + UMP	Purines: Pyrimidines
	UMP[a]	GMP	AMP	CMP			
4–6 S	25.5	28.2	24.5	21.8	50.0	1.00	1.11
18 S	24.1	32.1	20.1	23.7	55.8	1.26	1.10
28 S	19.5	36.5	16.8	27.2	53.7	1.76	1.14
DNA[b]	28.4	21.4	28.6	21.5	42.9	0.75	1.00

[a] In the case of DNA, this value represents mole per cent of TMP. [b] The nucleotide composition of DNA has been extracted from the data of Wyatt (1951). [c] Each value represents the average of three experiments. The methodology is presented in the text.

acid, the maximum specific activity of 1200 cpm/A_{254} was observed in the 45S region. The peak of radioactivity between the 18–28 and the 35 S exhibited the next highest specific activity (600 cpm/A_{254}).

The base compositions of the 4–6S, 18S, and 28S fractions of fetal liver nuclear RNA were determined after collection of suitable amounts from several sedimentation analyses and resedimentation in sucrose gradients. These data are presented in Table I. The per cent GMP plus CMP of the 4–6S, 18S, and 28S RNA was 50.0, 55.8, and 63.7%, respectively; the GMP plus CMP:AMP plus UMP was 1.00, 1.26, and 1.76. The base composition of DNA as determined by Wyatt (1951) is also presented in the table for comparison. The nucleotide compositions of the 18S and 28S fractions of fetal liver nRNA agreed quite favorably with the values obtained by Hirsch (1966) for 18S and 28S rRNA from several rat tissues.

Prior to determining the template activity of the nRNA, the requirements of the *E. coli* amino acid incorporating system were investigated. The *E. coli* system was completely dependent upon the presence

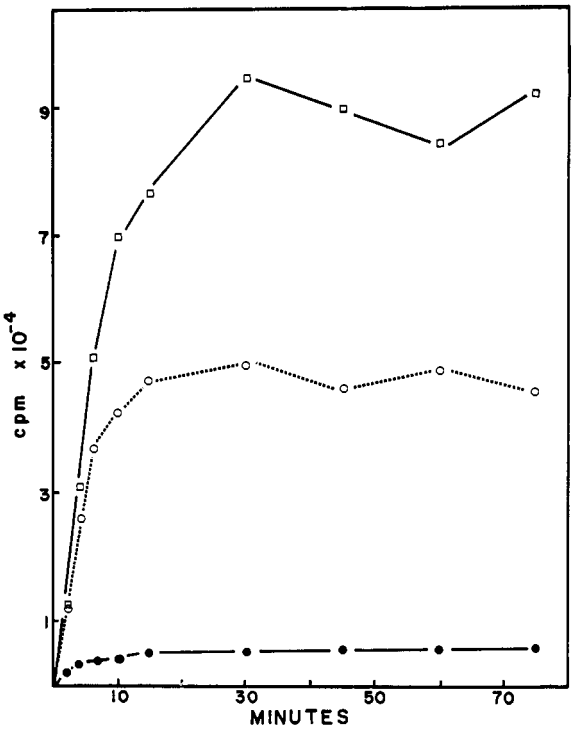

FIGURE 3: Stimulation of [^{14}C]phenylalanine incorporation by poly U. The *E. coli* amino acid incorporating system is described in the Methods and Materials section. The total counts per minute in the polypeptide is indicated on the ordinate. (●——●) Endogenous activity in the absence of any messenger. (○----○), 20 μg of poly U. (□——□), 50 μg of poly U.

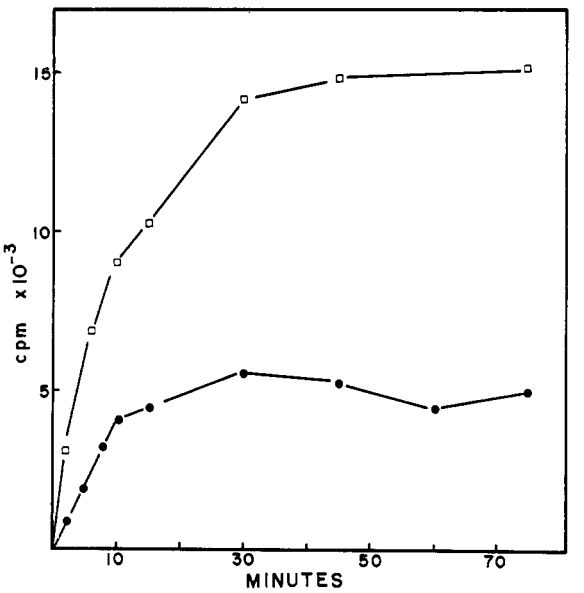

FIGURE 4: Kinetics of incorporation of [^{14}C]valine as stimulated by fetal liver nRNA. The experimental details are described in the text. Fetal liver nRNA (200 μg) was used in the assay (□——□). The total counts per minute incorporated into ^{14}C polypeptide is indicated on the left ordinate. (●——●) Endogenous activity.

TABLE II: Inhibition of Amino Acid Incorporation as Stimulated by Fetal Liver nRNA.[a]

Inhibitor (μg)	[^{14}C]Valine Incorp		Δ (cpm)	Inhibn (%)
	Endogenous	+ nRNA (cpm)		
None	4,780	11,400	6,620	–
Chloramphenicol (100)	1,220	1,480	260	96
Puromycin (100)	890	810	−80	100
Cycloheximide (100)	4,380	12,400	8,020	0
Streptomycin (50)	3,840	8,330	4,490	32
RNase (100)	525	535	10	100

[a] The constitution of the assay system is presented in the Materials and Methods section. The amount of fetal liver nRNA added to the 0.25-ml system was 64 μg.

of either a synthetic messenger, *i.e.*, poly U, homologous or a heterologous nRNA. The kinetics of the incorporation of labeled phenylalanine in the presence of poly U are indicated in Figure 3. A linear dependence was recorded for 5 or 10 min with 20 or 50 μg of poly U, respectively. Maximum stimulation was obtained at approximately 15–20 min with either amount of the synthetic messenger.

The kinetics of the incorporation of labeled valine into polypeptide material in the presence of fetal liver nRNA are depicted in Figure 4. Maximal stimulation was observed after 30 min of incubation. An almost linear dependence upon time was noted during 10 min of incubation.

The template activity of poly U, fetal, and adult nRNA is recorded in Figure 5. With this system, a linear dependence was observed between the amount of fetal or adult liver nRNA (up to at least 50 μg) and the μμmoles of valine incorporated/mg of S-30 protein. Maximal stimulation of phenylalanine incorporation was noted with 100 μg of poly U.

The effects of various inhibitors of protein synthesis upon the stimulation of amino acid incorporation by fetal liver nRNA has been determined (Table II). Ribonuclease completely abolished both the endogenous and the nRNA-stimulated incorporation of valine into polypeptide material. The antibiotics, puromycin and chloramphenicol, also exhibited a similar inhibition while cycloheximide was without effect. These data suggest that cycloheximide inhibition of protein synthesis will not occur in the presence of *E. coli* ribosomes even if a mammalian messenger is supplied to the system. Streptomycin inhibited the utilization of the labeled amino acid by 32%.

The addition of neomycin at concentrations below 1 μg/0.5 ml of test system to the *in vitro* protein-synthesizing system from *E. coli* enhanced the template activity of fetal liver nRNA (Table III). Holland and coworkers (1966) have also noted an increase in the template activity of r- and tRNA upon the addition of neomycin. Cleavage of a number of phosphodiester bonds of these RNAs by extensive heating was required prior to eliciting this effect. In the experiments reported here, an even greater stimulation of template activity in the presence of neomycin was observed upon the addition of boiled fetal liver nRNA, *i.e.*, 50% above control levels as opposed to 30% for the unboiled preparation. The efficacy of the boiled fetal liver RNA in the absence of the antibiotic, however, was less than with the unboiled preparation. At amounts of neomycin in excess of 1 μg, a progressive inhibition of

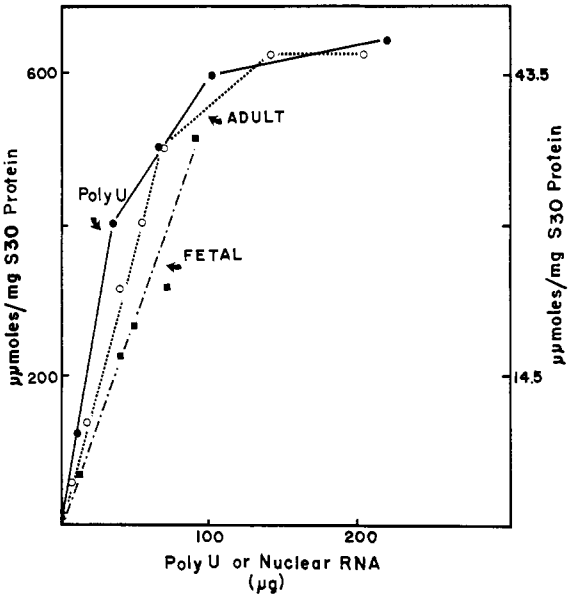

FIGURE 5: Stimulation of [^{14}C]amino acid incorporation by poly U, fetal, and adult liver nRNA. The experimental details are described in the text. The stimulatory activity of poly U (●——●) has been tested with [^{14}C]-phenylalanine (left ordinate) as the substrate while the template efficacy of dialyzed adult (○----○) or fetal liver (■—·—■) nRNA was determined with [^{14}C]-valine (right ordinate). The endogenous contribution has been subtracted from the experimental results. One micromicromole of [^{14}C]phenylalanine or [^{14}C]-valine represents 36.7 and 457 dpm, respectively.

TABLE III: Effect of Neomycin upon the nRNA-Stimulated Incorporation of [^{14}C]Valine.[a]

Amt Added	[^{14}C]Valine Incorpn		
	Endogeneous	+nRNA (cpm)	Δ (cpm)
	Expt A		
None	4,780	11,400	6,620
1	6,980	15,600	8,620 (130)[b]
5	5,490	9,860	4,370 (67)
45	1,760	2,350	590 (9)
	Expt B		
None	4,780	9,610	4,830 (100)
1	6,980	14,200	7,220 (150)
5	5,490	8,880	3,390 (90)
45	1,760	2,670	910 (19)

[a] The procedure is indicated in the Methods and Materials section. The fetal liver nRNA (64 μg) was added to the incubation system either before (expt A) or after (expt B) boiling at 100° for 5 min. [b] Per cent of the stimulatory activity (Δ).

protein synthesis was noted in all systems (Table III). The magnitude of the inhibition was similar with either boiled or unboiled fetal liver nRNA.

The efficacy of the fractions of fetal liver nRNA as templates in the *in vitro* synthesizing system was determined using sucrose density sedimentation as the means for their separation. Representative data are depicted in Figure 6. Little messenger-like activity was demonstrable in the 4–10S region of the gradients and indeed, oftentimes, an inhibition was observed. This inhibition was much more severe with the corresponding fraction from adult liver nRNA (Figure 7). Reports of the inhibition of amino acid incorporation by heterologous tRNA have also appeared from Korner's laboratory (Aaronson *et al.*, 1966). These investigators have reported that liver RNA inhibited the stimulation of [^{14}C]leucine incorporation into protein in the *in vitro E. coli* system brought out by liver microsomal RNA or poly U as template. The inhibitory effect appeared to be associated with the fraction of RNA possessing transfer activity. On the other hand, the stimulation of [^{14}C]phenylalanine incorporation by poly U or by liver microsomal RNA was not affected by RNA.

In our experiments, little messenger-like activity

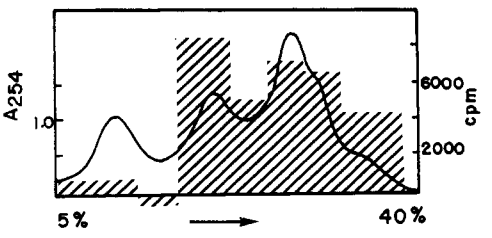

FIGURE 6: Template activity of fetal liver nRNA. Approximately 25 A_{260} units of fetal liver nRNA was layered over 5–40% linear sucrose gradients. Fractions from the gradient were combined as indicated in the figure and were then assayed for template activity as described in the text. The solid line represents the A_{254} (left ordinate) as determined in the Isco fractionator and ultraviolet absorption monitor while the diagonal lines represent the total counts per minute of [^{14}C]-valine incorporated into polypeptide (right ordinate). The direction of sedimentation is indicated by the arrow.

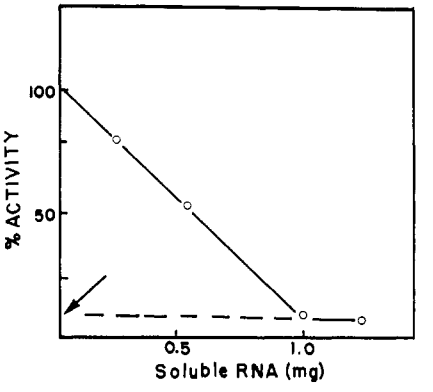

FIGURE 7: Inhibition of poly U stimulated [^{14}C]phenylalanine incorporation by RNA. RNA was added in the amounts indicated in the figure to the *E. coli* amino acid incorporating system in the presence of [^{14}C]-phenylalanine and 75 μg of poly U. The extent of [^{14}C]polyphenylalanine formation was determined and the per cent of the activity in the absence of the RNA was calculated (ordinate). The endogenous activity, *i.e.*, absence of poly U, is indicated by the arrow at the ordinate.

was found in the 6–10S regions of the gradient. The greatest specific stimulatory activity (per A_{254}) was noted in the 18S and 45S portions of the gradient. Considerable template activity was also noted in the region between 18 and 28 S.

Discussion

During the early fetal development, the mammalian liver is characterized by a period of extremely active cell division (Doljanski, 1960). It is during this phase that the liver mass is assembled. In the later stages of fetal liver development and continuing shortly after parturition, a phase of differentiation takes place during which specific cell products are elaborated. The pace of cell proliferation has diminished by this time although the cell size is continuously enlarging. These developmental stages may be correlated with the alterations in ploidy that accompany liver differentiation (Helweg-Larsen, 1952).

The following question arises. How is this alteration in ploidy which occurs during liver differentiation reflected in the synthesis of RNA and in the template activity of the nRNA? In a broad sense, this problem is related to the appearance of specific template RNA molecules during normal ontogenesis. The studies of Baserga *et al.* (1966) and Church and McCarthy (1967) bear upon this point. The former investigators have studied the rates of RNA synthesis in the livers of growing and adult mice, *i.e.*, 12-days and 5-months old, respectively. When proper consideration is given to the extent of the precursor pool size, the rates of RNA synthesis were higher in the neonatal than in the adult tissue. A similar finding had been observed by Oliver and Blumer (1964).

Church and McCarthy (1967) have examined the synthesis of RNA in fetal mouse liver in the period from the 14th day of gestation until parturition. By means of hybridization techniques, they have established the synthesis of characteristic RNA populations during the various stages of liver development. The most significant difference was observed at the very early times, at 14 days of fetal development. In their studies, the 14-day embryonic liver RNA resembled early regenerating liver RNA.

The results presented here indicate the feasibility of studying epigenetic transitions occurring in nuclei during the development of fetal rat liver. The major contribution of this study is in ascertaining the distribution of template RNA activity in fetal liver nuclei by combined sedimentation and *E. coli* amino acid incorporating techniques. A number of investigators have employed the latter in localizing the messenger-like activity of a liver nRNA preparation (Barondes *et al.*, 1962; Brawerman *et al.*, 1963; Revel and Hiatt, 1964; DiGirolamo *et al.*, 1964; Cartouzou *et al.*, 1965). DiGirolamo *et al.* (1964) have demonstrated the heterogeneity of stimulatory nRNA although a peak of maximum activity was noted in the 18S region. Hadjivassiliou and Brawerman (1967) have succeeded in extracting RNA fractions from rat liver nuclei at alkaline pH which are very rapidly labeled and which exhibit high template activity. The template material sedimented between 9 and 16 S and had a DNA-like base composition.

Our results indicate the heterogeneity of template activity in fetal liver nuclear preparations. Maximal messenger activity, *i.e.*, specific activity, was observed in the 18S and 45S regions of the sedimentation gradient. These regions were also substantially labeled after an *in vivo* pulse with orotic acid. A considerable amount of template activity and rapidly labeled nRNA is present in the gradient in regions in excess of 28 S. These findings are in accord with the results of DiGirolamo *et al.* (1964) with normal rat liver but differ from those of Hadjivassiliou and Brawerman (1967).

A word of caution about the interpretation of the results obtained from the amino acid incorporation system would be in order. It has been postulated by Roberts (1965), Osawa (1965), and Yoshikawa-Fukada (1966) that rRNA when in an unmethylated, *i.e.*, nascent, state may possess template properties for the construction of ribosomal proteins. When the RNA is incorporated into the mature ribosomal particle, methylation and the loss in template activity occur. Recently, however, evidence has been forthcoming from several laboratories (Manor and Haselkorn, 1967) (Sypherd, 1967) which does not favor this hypothesis. The message activity is believed to reside in normal mRNA which contaminates the rRNA preparation. Although the data presented in this report is in agreement with either hypothesis, we would lean toward the latter.

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Limited Enzymatic Addition of Deoxyribonucleotide Units onto Chemically Synthesized Oligodeoxyribo-5′-nucleotides*

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ABSTRACT: The oligomer-initiated polymerization of deoxyribonucleotide units catalyzed by terminal deoxyribonucleotidyl transferase has been limited by reaction stoichiometry to the formation of short polymers. The product distributions conform reasonably well to Poisson statistical theory in these cases. However, in the self-limiting polymerization of deoxyguanylate units, the distribution was very sharp.

Oligo- and polydeoxyribonucleotides of varied and known structure are miniature models of DNA suitable for studies of its chemical and biological properties. Such compounds have been synthesized by exclusively chemical procedures (Jacob and Khorana, 1965) and also by combinations of chemical and enzymatic methods (Bollum *et al.*, 1964; Byrd *et al.*, 1965; Hayes *et al.*, 1966).

The calf thymus derived enzyme, terminal deoxyribonucleotidyl transferase (hereafter referred to as addase), has been used to effect syntheses of polydeoxyribonucleotides with 100 or more monomer units of one kind (Bollum *et al.*, 1964) or with mixed sequences of different kinds of monomers (Ratliff *et al.*, 1967). This reaction involves repetitive grafting of mononucleotide units from a deoxyribonucleoside 5′-triphosphate onto an oligodeoxyribonucleotide initiator beginning at its terminal 3′-hydroxyl. Before addase had been separated from calf thymus DNA polymerase (Bollum *et al.*, 1964), Bollum (1962) had used the partially purified mixture of these enzymes to add just a few nucleotide units onto various initiators.

We have used addase freed of DNA polymerase (Yoneda and Bollum, 1965) to effect synthesis of short polynucleotides by using small ratios of deoxyribonucleoside 5′-triphosphate to initiator. In the case using dGTP,[1] an additional self-limiting effect on length has already been noted (Ratliff and Hayes, 1967) at greater than elevenfold ratios of triphosphate to initiator. Distributions of products formed during a reaction have been compared with Poisson theory.

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[1] Abbreviations used: oligodeoxynucleotides are designated by the usual capital letters followed by subscripts representing the number of units. Examples are T_6 hexamer of 5′-thymidylic