ISOPRENYLATION OF TRANSFER RIBONUCLEIC ACID*

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SUMMARY. A two-fold difference in the total N^6-(Δ^2-isopentenyl) adenosine content was found between the serine accepting tRNA fractions from adult and embryonic bovine liver. Elution profiles of benzoylated DEAE cellulose showed three peaks of adult tRNA were capable of accepting serine. Using gas-liquid chromatography, each peak had measurable amounts of N^6-(Δ^2-isopentenyl) adenosine. When the same techniques were applied to embryonic bovine tRNA, three peaks accepted serine; however, only one peak contained N^6-(Δ^2-isopentenyl) adenosine. These results can be interpreted to indicate that adult and embryonic tRNA differ in the N^6-(Δ^2-isopentenyl) adenosine content of $tRNA^{ser}$.

Analysis of RNA hydrolysates provided the first indication of the existence of modified bases in the protein synthetic apparatus of biological systems (1, 2, 3). Subsequently, 30 modified nucleosides, comprising 10-20% of the total, have been isolated and identified from mammalian, plant, and bacterial tRNA sources (4). N^6-(Δ^2-isopentenyl) adenosine and other modified forms of this nucleoside have been identified in isoaccepting species of tRNA which bind a condon beginning with U, and N-(nebularin-6-ylcarbamoyl) threonine has been determined in those isoaccepting tRNA species which recognize a codon beginning with the letter A (5).

Differences in the protein biosynthetic apparatus between embryonic and adult organs have been illustrated by differences in tRNA methylation and amino acylation (6, 7). In this investigation, differences between the isopentenyl adenosine modification in embryonic and adult bovine liver tRNA were examined.

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Possible implications relative to embryonic development and protein biosynthesis are suggested.

MATERIALS

Embryonic bovine liver was obtained from Pel-Freeze Biologicals, Inc., Rogers, Ark., and fresh adult bovine liver was obtained from the Department of Animal Sciences at the University of Florida. The following materials were commercially available: Benzoylated DEAE Cellulose, Schwarz Bio Research; ^{14}C-Serine (126-218 mc/mm), Aquasol, New England Nuclear; snake venom from *Crotalus adamanteus* (Eastern Diamondback Rattlesnake); RNAase T_1 from *Aspergillus orgazae*; alkaline phosphatase from *E. coli* (Type III-S), Sigma Chemical Company; Aminex A-6, Bio-Rad Laboratories; BSTFA [bis(trimethylsilyl)trifluoroacetamide] with 1% TMCS, Pyridine, silylation grade, Reacti-vials (0.3 mls-total cone volume), Pierce Chemical Company.

The following buffer systems were used for adult and embryonic tRNA preparation and chromatography: Buffer A: 0.01 M Tris-HCl (pH 8.0), 0.01 M $Mg(OAc)_2$, 0.05 M KCl, 0.60 M sucrose, 0.001 M β-mercaptoethanol, 0.001 M EDTA·2Na and 1.0 mg ml^{-1} bentonite; Buffer B: 0.01 M Tris-HCl (pH 7.5) 0.01 M $Mg(OAc)_2$, 0.15 M NaCl, 0.001 M β-mercaptoethanol, 0.001 M EDTA $(Na)_2$ and 2.0 mg ml^{-1} bentonite; Buffer C: 0.01 M NaOAc (pH 4.5) 0.01 M $MgCl_2$ and 0.001 M β-mercaptoethanol.

PROCEDURES

Isolation of tRNA

Equal parts of liver and buffer A (w/v) were homogenized in a Sorvall Omnimixer at 10,000 rpm for 5 minutes. Unless indicated, all procedures were performed at 0-4°C. The homogenate was centrifuged at 650 xg for 15 minutes, 10,5000 xg for 15 minutes, and 20,500 xg for 15 minutes. An equal volume of Buffer B was added to the supernatant, followed by an equal volume of phenol-water (15:3 v/v) containing 0.1% 8-hydroxyquinoline to remove protein components. The aqueous and phenol layers were separated by centrifugation at 6,000 xg.

The separated aqueous phase was adjusted to 1 M NaCl. Following incubation for 12-16 hrs. at 4°C, the solution was centrifuged at 6,000 xg for 15 minutes. The supernatant was adjusted to 70% ethanol by addition of absolute ethanol and refrigerated at 4°C for 12 hrs. The resulting precipitate was collected by centrifugation at 6,000 xg for 15 minutes. The dried precipitate was dissolved in Buffer C containing 0.30 M NaCl and stored at -20°C until benzoylated DEAE cellulose chromatography could be accomplished.

Benzoylated DEAE Cellulose Chromatography

Adult and fetal calf RNA were applied to benzoylated DEAE cellulose columns in Buffer C containing 0.30 M NaCl. Elution was accomplished with a linear 0.30 M to 1.00 M NaCl gradient. The final RNA fraction was removed with Buffer C containing 1.00 M NaCl -20% EtOH. The flow rate was approximately 60 ml/hr and the column temperature was 22°-24°C (8).

The benzoylated DEAE cellulose chromatography RNA fractions were precipitated by addition of 2 1/2 volumes of absolute ethanol and collected on millipore filters. The precipitates were eluted from the filters with 0.01 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.5) by agitation and stored at -20°C until they were assayed.

The serine accepting fractions were combined. RNA precipitated with 2.5 volumes of absolute ethanol was reisolated and dried *in vacuo* at room temperature.

Serine tRNA acceptor activity was measured using the following assay mixture: 0.04 mM Tris-HCl (pH 7.4); 0.0066 M $MgCl_2$; 0.0025 M ATP; amino acyl synthetase (80 µg protein); variable amounts of the tRNA fractions; and 10 mM ^{14}C-serine (sp. act. 126-128 miC mM^{-1} in a final volume of 0.30 ml). The adult amino acyl synthetase was prepared by the method of Hatfield and Portugal (9).

At designated times 50 to 100 microliter aliquots of the assay mixture were placed on 1 inch square Whatman 3MM filters, air dried, and placed in about 10 ml cold 10% TCA. The filters were then washed sequentially with 10 ml of the following solutions: cold 5% TCA for 15 minutes (2 times); Hokins

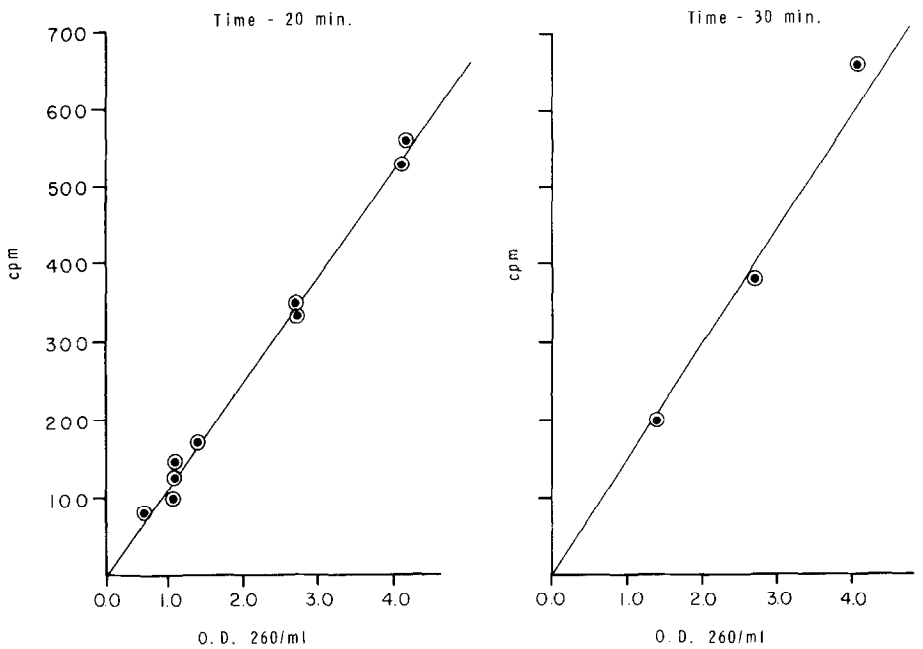

Figure 1. Serine incorporation (cpm) vs. O.D.$_{260}$ unit/ml assay adult tRNA ([Mg++]/[ATP] = 3.0)

solution for 20 minutes at 37°C; equal volumes of Hokins solution and ether for 10 minutes at room temperature; and ether for 20 minutes at room temperature. The washed filters were air dried, vigorously agitated in 10 ml of aquasol scintillation fluid and counted.

Linearity between ^{14}C-serine incorporation and adult tRNA concentration was observed (Figure 1); similar linearity was obtained using embryonic tRNA. The serine accepting activity of each of the fractions from benzoylated DEAE cellulose chromatography was measured using the same concentration of tRNA (1.0 A_{260} unit ml^{-1}) in all the assay mixtures.

Aminex A-6 Chromatography

The dried tRNA fractions were dissolved and treated initially with RNAase T_1 from Aspergillus orgazae (0.1 mg/ml) at 37°C for 1 hour (10). Snake venom diesterase (1 mg $mg_{t_{RNA}}$) and alkaline phosphatase (21 units as defined by Sigma) were then added and digestion continued at 45°C for 12 hours (10). The digestion mixture was lyophilized and 1 ml of 0.85 M ammonium acetate (pH 5.70)

was added to the dried precipitate. This solution was placed on an Aminex A-6 column (0.6 cm x 20 cm) equilibrated in 0.85 M ammonium acetate (pH 5.70). The flow rate was 7-15 ml/hr and the column temperature was 40°C.

After the major nucleosides and other materials were eluted from the column, the isoprenylated nucleosides were eluted with 0.85 M ammonium acetate buffer (pH 5.70) containing 10% ethanol.

Fractions collected from the ammonium acetate-ethanol wash were combined into sets of approximately 10 tubes. Each pooled fraction was extracted 4 times with 5 ml aliquots of ethyl acetate (11). The ethyl acetate layer was taken to dryness under a vacuum.

Silylation and Gas Chromatography Analysis of N^6-(Δ-isopentenyl) Adenosine

The isoprenylated adult tRNA in the lyophilized reacti-vials was silylated in 0.01 ml silylation grade pyridine and 0.030 ml BSTFA containing 1% TMCS. In the case of embryonic tRNA, 0.003 ml pyridine and 0.007 ml BSTFA containing 1% TMCS were used. Silylation was continued for 30 minutes at 80°-85°C (12). Aliquots of 1-5 microliters were gas chromatographed at 280°C (isothermal) on a 3% SE-30 on Chromosorb G (80/100 mesh) column using a Hewlett-Packard Model 402 B Gas Chromatograph.

RESULTS

Serine accepting activity ($tRNA^{ser}$) of both adult and embryonic tRNA was resolved into major peaks using chromatography on benzoylated DEAE cellulose (Figure 2). These results are consistent with similar data using rabbit, adult bovine, rat, and rooster liver tRNA (9, 13, 8). This chromatographic method resulted in peaks which showed a two- to eleven-fold enrichment of adult serine accepting activity compared to the sample of mixed tRNA which was initially applied to the column.

Fractions resulting from benzoylated DEAE chromatography of both embryonic and adult tRNA were combined into 3 major fractions (Figure 2), reassayed for total serine accepting activity, enzymatically hydrolyzed to the nucleosides chromatographed on Aminex A-6 resin, and analyzed for N^6-(Δ^2-isopentenyl) ade-

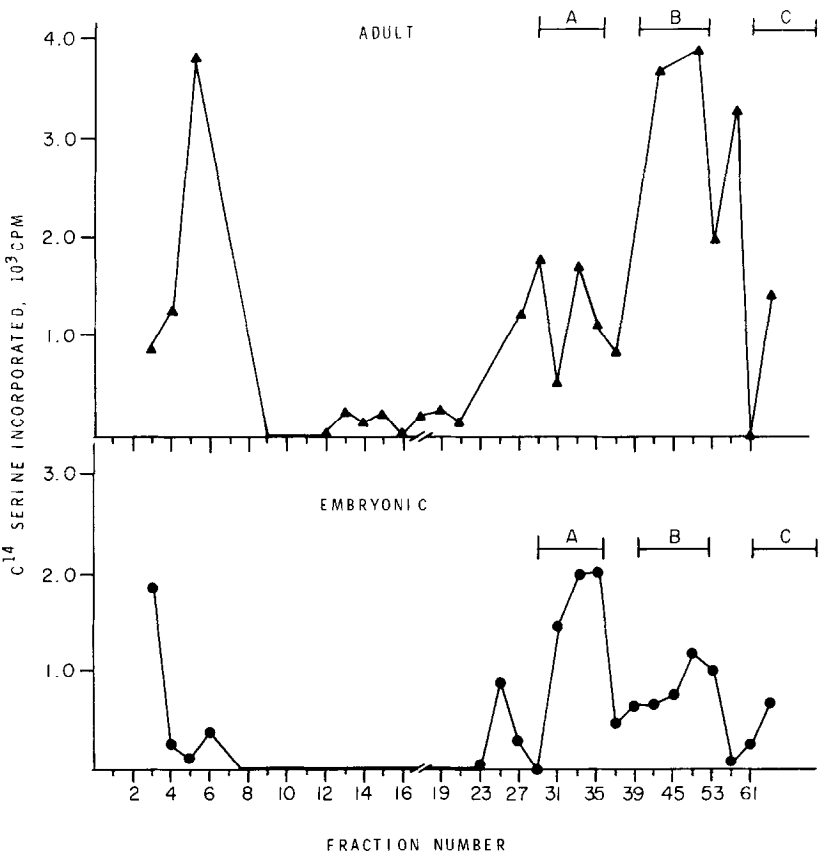

Figure 2. Benzoylated DEAE cellulose chromatography of adult and embryonic bovine tRNA. Column dimensions and volume of the elution gradient were 2 x 31 cm, 900 ml, and 2 x 57.5 cm, 1500 ml for adult and embryonic tRNA, respectively.

nosine content by gas-liquid chromatography. Repeat studies of fraction A indicate that it is homogeneous with respect to serine accepting activity. Fraction C of Figure 2 consists of tubes 61-69. Table 1 shows total tRNA (A_{260} units), serine accepting activity, and the theoretical amount of N^6-(Δ^2-isopentenyl) adenosine in each of these pooled fractions. The theoretical amount of N^6-(Δ^2-isopentenyl) adenosine represents the minimum amount to be detected by gas-liquid chromatography and was estimated from the molecular weight of N^6-(Δ^2-isopentenyl) adenosine and the total moles of serine accepting activity of each fraction, assuming that each mole of $tRNA^{ser}$ contains a minimum of 1 mole of N^6-(Δ^2-isopentenyl) adenosine.

Table 1. tRNA content, serine accepting activity, and calculated amounts of N^6-(Δ^2-isopentenyl) adenosine present in combined fractions from benzoylated DEAE cellulose chromatography and experimentally found N^6-(Δ^2-isopentenyl) adenosine.

Fraction	tRNA* (moles x 10^7)	Serine Incorporated (moles x 10^{10})	Calculated** 6 ip Ado (g x 10^9)	6 ip Ado*** (g x 10^6)
Adult				
A****	3.3	11	362	0.08±0.01
B	1.4	8.6	288	1.0±0.20
C	2.6	6.7	225	11.4±1.9
Embryonic				
A	0.5	1.8	60	Not Detected
B	1.0	2.7	91	Not Detected
C	2.9	5.4	181	3.2±0.5

*Calculated from the total A_{260} units assuming that 20 A_{260} units = 1 mg tRNA and the molecular weight of tRNA = 30,000 $gmole^{-1}$.

**Calculated from total moles of serine incorporated and the molecular weight of N^6-(Δ^2-isopentenyl) adenosine (6 ip Ado) assuming that each mole of $tRNA^{ser}$ contains a minimum of 1 mole of 6 ip Ado.

***Corrected assuming 91±13% recovery of the sample applied.

****Fractions A, B, and C represent the equivalent fractions shown in Figure 2 resulting from DEAE cellulose chromatography.

Standard samples of N^6-(Δ^2-isopentenyl) adenosine were subjected to Aminex A-6 and gas-liquid chromatography. Recoveries ranged from 75-111% with a mean of 91±13%. Thus, this method was judged sufficient to determine semiquantitatively the amount of N^6-(Δ^2-isopentenyl) adenosine in the fractions from benzoylated DEAE cellulose chromatography. These results (Table 1) indicate that N^6-(Δ^2-isopentenyl) adenosine is found in all the adult tRNA fractions but in only one of the serine accepting fractions from the embryonic source. Absence of N^6-(Δ^2-isopentenyl) adenosine in embryonic fraction B is clearly demonstrated because the total moles of tRNA subjected to analysis was comparable to the moles analyzed in adult fraction B. In the case of embryonic fraction A, failure to detect N^6-(Δ^2-isopentenyl) adenosine may be due to the fact that the amount of tRNA available for analysis was only 15% of that analyzed in adult fraction A.

In the case of all the adult liver tRNA fractions and in fraction B of the embryonic tRNA, the total amount of N^6-(Δ^2-isopentenyl) adenosine is considerably greater than the amount expected, based on serine accepting activity (Table 1). This suggests that another amino acid accepting tRNA is present in these fractions. Since microbial $tRNA^{tyr}$ contains N^6-(Δ^2-isopentenyl) adenosine (14), the tyrosine accepting activity of both the adult and embryonic benzoylated DEAE cellulose fractions was measured. Data shown in Table 2 indicate that total serine plus tyrosine accepting activity more closely accounts for the moles of N^6-(Δ^2-isopentenyl) adenosine found.

These studies have been repeated on three separate occasions, indicating that two of the three embryonic serial tRNAs do not contain the isopentenyl modification. These tRNAs are now being used as substrates for the study of the isopentenyl transferase.

DISCUSSION

Detection of N^6-(Δ^2-isopentenyl) adenosine in adult and embryonic bovine liver tRNA is consistent with previous results where N^6-(Δ^2-isopentenyl) adenosine was obtained from calf liver and chick embryo tRNA. The isopentenyl side

Table 2. Total serine plus tyrosine accepting activity of adult and embryonic tRNA fractions from benzoylated DEAE cellulose chromatography.

Fraction	Serine Plus Tyrosine Incorporated (mole x 10^9)	6 ip Ado* (mole x 10^9)
Adult		
A	0.21	0.24
B	3.0	3.0
C	9.3	3.4
Embryonic		
A	0.26	0
B	1.5	0
C	7.3	9.5

*Calculated from data in Table 1.

chain is attached to an adenylate residue in the preformed tRNA molecule (11, 15, 16, 17, 18) by the enzyme, Δ^2-isopentenyl-pyrophosphatase: Δ^2-isoprenylated tRNA sequence, A-A-A-Ψ-C (19-23).

Results of this work show that embryonic tRNA fraction B is clearly deficient in N^6-(Δ^2-isopentenyl) adenosine when compared to the corresponding adult fraction. The data also suggest N^6-(Δ^2-isopentenyl) adenosine may be absent from embryonic tRNA fraction A.

This difference is further illustrated by comparison of the ratios of the total moles of N^6-(Δ^2-isopentenyl) adenosine divided by the total moles tRNA for all of the benzoylated DEAE cellulose fractions from both adult and embryonic bovine liver tRNA. This ratio was 0.05 for adult tRNA and 0.02 for embryonic, thus indicating an approximate 2.5-fold decrease in the extent of total embryonic tRNA isoprenylation compared to the adult.

Since the isopentenyl side chain is involved in tRNA binding to the m-RNA-ribosome complex, the isoprenylation of the adenosine adjacent to the anticodon could function in the regulation of protein synthesis. Differences in the degree of isoprenylation may suggest differences in either the specificity or the amount of Δ^2-isopentenyl pyrophosphate: Δ^2-isoprenylated-tRNA transferase

at specific levels of differentiation and, hence, a possible regulatory function in the translation mechanism of nucleic acid to protein.

Existence of both an adult and embryonic serine accepting tRNA in a fraction (fraction C) which requires ethanol (1.0 M NaCl) for elution from benzoylated DEAE cellulose is consistent with results obtained from rooster (8) and adult bovine liver tRNA (9). Unique features of the $tRNA^{ser}$ are the phosphorylation of seryl-$tRNA^{ser}$ and recognition of the termination codon UGA (8, 9). Its role in protein synthesis is presently unknown; however, the results imply a similar $tRNA^{ser}$ in adult and embryonic tRNA. Further purification of mammalian isoaccepting tRNA for tyrosine, serine, and phenylalanine is required to determine whether this hypothesis is applicable to embryonic and adult tRNA.

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