MOLECULAR AND CELLULAR BIOLOGY, Mar. 1982, p. 241-249
0270-7306/82/030241-09$02.00/0

Vol. 2, No. 3

Nuclear Ligation of RNA 5'-OH Kinase Products in tRNA

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Received 11 August 1981/Accepted 19 October 1981

Mouse L-cell nuclei incorporate γ-^{32}P from ATP in vitro predominantly in 5'-monophosphoryl termini and internal phosphodiester bonds with a nonrandom nearest-neighbor distribution. In the presence of 1 μg of α-amanitin per ml the γ-^{32}P showed a time-dependent appearance in RNA bands which migrated with mature tRNA species but not with pre-tRNA and 5S RNA. The γ-^{32}P was found in internal phosphodiester bonds as shown by alkaline phosphatase resistance and was identified in 3'-monophosphates after RNase T_2, T_1, and A digestion. The specificity of this incorporation was indicated by a limited number of labeled oligonucleotides from a T_1 digest and identification of 70 to 80% of the ^{32}P label as Cp on complete digestion of the eluted tRNA band. We also observed transiently [γ-^{32}P]ATP-labeled RNA bands (in 5'-monophosphate positions) that were 32 to 45 nucleotides long. The results presented suggest splicing of several mouse L-cell tRNA species in isolated nuclei which involve the RNA 5'-OH kinase products as intermediates.

Transfer RNA gene expression has been investigated extensively for the study of a variety of RNA processing steps both in vivo and in vitro (see reviews in references 1 and 2). The presence of intervening sequences in several yeast tRNA genes (8, 9, 11, 16, 21), as well as *Drosophila* tRNA genes (9), has recently made the eucaryotic tRNA system also useful in the study of splicing reactions and their role in the overall tRNA processing scheme (6, 12, 15-17, 19).

The splicing reaction in tRNA processing has been shown to consist of two identifiable steps (12, 19) in yeast, and two distinct enzyme activities involved in splicing have been also detected in *Xenopus* oocyte nuclei (15, 18). The half-tRNA molecules which result from the cleavage reaction have 3'-phosphate and 5'-hydroxyl termini (12, 18). The mechanism of the subsequent ligation step is less well characterized, but has a strict requirement for ATP (19).

Characterization in our laboratory of RNA products radiolabeled in isolated nuclei by endogenous polynucleotide kinase (22) showed the appearance of some γ-^{32}P from ATP in internal phosphodiester bonds (Winicov, unpublished data), suggesting that the kinase products may be intermediates in RNA ligation reactions. A sizable proportion of both RNA polynucleotide kinase products and the proposed RNA ligase products were recovered in the 12-3S RNA size class. Although there have been no reports to date on spliced tRNA species in mammalian cells, we investigated the labeling of endogenous tRNA by [γ-^{32}P]ATP in isolated nuclei. We were able to show rapid specific labeling of the 5'-monophosphate groups on molecules in the range of half tRNA size with subsequent incorporation of the γ-^{32}P from ATP in internal phosphodiester bonds of tRNA-sized RNA. These results suggest the ligation of several mammalian tRNA species involving the 5'-phosphoryl termini of RNA polynucleotide kinase products.

MATERIALS AND METHODS

Nuclear RNA labeling and isolation. The α-amanitin was purchased from Sigma Chemical Co. (St. Louis, Mo.). ^{3}H-labeled UTP, [γ-^{32}P]ATP, and α-^{32}P-labeled ATP and UTP were obtained from New England Nuclear Corp. (Boston, Mass.) and used at concentrations given for individual experiments.

DNase I (Boehringer-Mannheim, Indianapolis, Ind.) and bacterial alkaline phosphatase (Worthington, Freehold, N.J.) were further purified by passage through an agarose 5'-(*p*-aminophenyl phosphoryl)uridine 2'-(3')-phosphate column (Miles Laboratories, Inc., Elkhart, Ind.) to remove contaminating RNase (10). Alkaline phosphatase eluted from the affinity column slightly behind DNase, but retained its activity during chromatography in sodium acetate buffer at pH 5.0. The eluted alkaline phosphatase peak was pooled and adjusted to pH 7.6 for storage and subsequent use. All digestions with the purified alkaline phosphatase were carried out at 50°C, using 0.2 U/ml.

Methods. Mouse L cells were treated with 80 ng of actinomycin D per ml, and the nuclei were prepared and incubated as described previously (23). However, to insure continuous [γ-^{32}P]ATP incorporation, it was necessary to keep the nuclear concentration at 7×10^7 to 9×10^7 per ml of incubation mixture, since high L-cell nuclear concentrations ($\sim 1.5 \times 10^8$/ml) rapidly deplete the ATP concentration needed for continued

incorporation. The conditions of RNA synthesis were as described (23), with GTP at 1.0 mM, CTP and ATP at 0.5 mM, and UTP at 0.3 mM. The reaction was stopped by quickly freezing the assay mixture in ethanol-dry ice.

The thawed nuclei were treated with a Tween-40–deoxycholate wash, lysed in high-salt buffer, and treated with affinity column-treated DNase I (23) as described previously. The pooled nuclear and wash fractions were extracted twice with chloroform-phenol, and the aqueous phase was precipitated with 2.5 volumes of ethanol. The extracted RNA was separated from mononucleotides on a Sephadex G-50 column as reported (24).

Electrophoretic RNA analysis. RNA was analyzed by tube gel electrophoresis on 10% acrylamide-bisacrylamide–6 M urea for 4 h at 5 mA per gel (11.5 cm) in Tris-acetate (pH 7.2). The gels were sliced, dissolved in Protosol (New England Nuclear Corp.), and counted in Omnifluor (New England Nuclear Corp.)-toluene. When gels were to be eluted, the sliced gel was monitored by Cerenkov counting. The gel slices for each band were pooled, minced, and eluted with 3 volumes of 0.3 M NaCl, 0.1 mM EDTA, 10 mM Tris-hydrochloride (pH 7.4) and 1% phenol for 30 min at 45°C (19). The mixture was underlayered with 1 ml of 60% glycerol and centrifuged to remove the minced gel. RNA in the supernatant fraction was precipitated with 2.5 volumes of ethanol for further analysis. The slab gel (325 by 150 by 0.75 mm) contained 10 or 20% acrylamide-bisacrylamide with a 25-mm top layer of 3.5% acrylamide, 6 M urea, 1 mM EDTA, and 50 mM Tris-borate (pH 8.5). Electrophoresis was for 14 h at 190 V and room temperature. Autoradiography of the dried gel was with Kodak No Screen X-ray film for 2 to 6 days at room temperature.

Two-dimensional gel electrophoresis was carried out using slab gel electrophoresis in the first dimension as described above. The tRNA region (as estimated by dye markers) was cut out of the 10% gel, and the excised gel was fitted on a 20% acrylamide-bisacrylamide–4 M urea (160 by 145 by 3 mm) gel and secured in place with the 20% acrylamide. Electrophoresis in the second dimension was at 2°C and 177 V for 15 h.

RESULTS

α-Amanitin sensitivity of in vitro RNA synthesis and ^{32}P incorporation from [γ-^{32}P]ATP. We had previously shown that the predominant L-cell transcription product in vitro is RNA transcribed by RNA polymerase II (23, 24). To determine RNA polymerase III activity in these nuclei we followed RNA synthesis in the presence of increasing doses of α-amanitin and measured [^{3}H]UMP incorporation in isolated RNA. After a 30-min incubation, 74% of total RNA accumulated was the product of RNA polymerase II (sensitive to 1 μg of α-amanitin per ml), and about 21% of the RNA was the product of the RNA polymerase III (sensitive to 100 μg of α-amanitin per ml) (Table 1). The same double label experiments also showed that the incorporation of γ-^{32}P from ATP is relatively little affected by the presence of α-amanitin even at 100-μg/ml concentrations (Table 1). These results suggest that γ-^{32}P incorporation from ATP is not highly dependent on continued RNA synthesis in isolated nuclei and may involve RNA molecules synthesized in vivo, i.e., preformed in intact cells.

Time course of in vitro RNA synthesis and γ-^{32}P incorporation from [γ-^{32}P]ATP in RNA polymerase III products. The most abundant RNA polymerase III products are 5S RNA and tRNA. They can be easily identified by gel electrophoresis, and their synthesis is inhibited by α-amanitin at 100 μg/ml. Since we have observed incorporation of γ-^{32}P from ATP in all RNA size classes (22), including internal phosphodiester bonds (Winicov, unpublished data), we compared the two major transcripts of polymerase III (5S and tRNA) in our attempts to identify specific RNA products labeled in isolated nuclei. To identify the specific endogenous RNA products labeled in nuclei with ^{32}P from [γ-^{32}P]ATP we followed the time course of ^{32}P incorporation as well as RNA synthesis (with [^{3}H]UTP or [α-^{32}P]ATP in the presence of 1 μg of α-amanitin per ml). To differentiate the γ-^{32}P from ATP incorporated at the 5′ end of molecules (either by initiation or RNA polynucleotide kinase) from ^{32}P incorporated in internal phosphodiester bonds, we treated samples of the extracted RNA with affinity-purified bacterial alkaline phosphatase and analyzed both treated and untreated samples by slab gel electrophoresis in 6 M urea as shown in Fig. 1.

The time course of labeling with [α-^{32}P]UTP shows increased accumulation with time of 5S and pre-tRNA. Very little [α-^{32}P]UTP was detected in the mature tRNA region after 5 min of labeling, but the intensity of the 4S band increased with time, indicating that the newly synthesized pre-tRNA molecules were being processed in nuclei. The γ-^{32}P from ATP appeared to show little if any incorporation in 5S

TABLE 1. In vitro RNA synthesis and ^{32}P incorporation from [γ-^{32}P]ATP: sensitivity to α-amanitin

α-Amanitin concn (μg/ml)	pmol incorporated[a]	
	[^{3}H]UMP	[γ-^{32}P]ATP
0	689	16
1[b]	163	14
10	142	10
100	35	13

[a] Incorporation in 30 min per 3 × 10^7 nuclei. [^{3}H]UTP was used at 0.3 mM (specific activity, 0.6 mCi/μmol); [γ-^{32}P]ATP was used at 0.5 mM (specific activity, 1.1 or 1.5 mCi/μmol). α-Amanitin was added at 0 time.

[b] Average of three experiments.

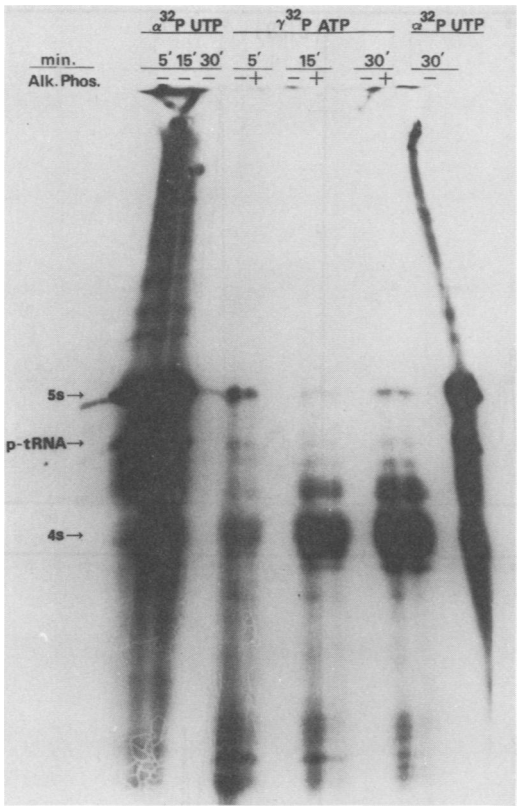

FIG. 1. Time-dependent [α-^{32}P]UTP and [γ-^{32}P]ATP incorporation in nuclear RNA in the presence of 1 μg of α-amanitin per ml. Nuclei were incubated at 37°C for 5, 15, or 30 min in the presence of [α-^{32}P]UTP (specific activity, 700 mCi/mmol, 0.3 mM) or [γ-^{32}P]ATP (specific activity, 1.7 Ci/mmol, 0.5 mM) plus [^{3}H]UTP (600 mCi/mmol, 0.3 mM) in a normal RNA synthesis system. The isolated RNA was separated by 10% acrylamide-bisacrylamide–6 M urea slab gel electrophoresis as shown, after one-half of each [γ-^{32}P]ATP-labeled sample was digested with alkaline phosphatase (0.2 U/ml) as described. Lanes marked (+) contain alkaline phosphatase-treated RNA. Autoradiography was for 6 days at room temperature. Arrows show positions of in vivo ^{32}P-labeled L-cell 5S and 4S RNA.

and pre-tRNA sized molecules. The small amount of radioactivity in this region appeared to be sensitive to alkaline phosphatase and may be due to newly initiated molecules. The low level of ^{32}P in 5S RNA is presumed to be due to the γ phosphorus exchange between nucleotide triphosphates (4; Winicov, unpublished data).

Most of the ^{32}P from [γ-^{32}P]ATP, however, was found in bands which comigrated with mature tRNA species and a number of distinct bands with apparent estimated sizes of 32 to 45 nucleotides. The ^{32}P in these short molecules is partially alkaline phosphatase sensitive. These molecules appeared to be the most highly labeled by [γ-^{32}P]ATP in the first 5 min of incubation, with a subsequent decrease in intensity during the 30-min incubation period. In contrast, the mature tRNA-sized RNA bands continued to be labeled with time by [γ-^{32}P]ATP, and the ^{32}P remained alkaline phosphatase resistant, indicating its position in internal phosphodiester bonds.

Selective degradation of the short RNA species by contaminating nuclease activity was ruled out by tube gel electrophoresis of duplicates of the samples presented in Fig. 1, doubly labeled with [γ-^{32}P]ATP and [^{3}H]UTP. Figure 2 shows this time dependence of γ-^{32}P incorporation from ATP in alkaline phosphatase-sensitive and -resistant forms of RNA, with tRNA-sized RNA completely resistant to alkaline phosphatase. The distinct 32- to 45-nucleotide-long bands seen in Fig. 1 migrated as a broad peak between mature tRNA and the bromphenol blue tracking dye. Although the ^{32}P counts in this band were alkaline phosphatase sensitive, the enzyme treatment did not alter the ^{3}H-labeled RNA profile, ruling out selective degradation by contaminating nucleases. Although the separation in Fig. 2 is not as good as in Fig. 1, the positions of 5S, pre-tRNA, 4S, and the small RNA molecules can be clearly differentiated. The position of pre-tRNA size molecules is identified in Fig. 3, where the pre-tRNA was labeled with *S*-[^{3}H]adenosylmethionine in vitro.

Verification of the ^{32}P position in RNA molecules. The position of ^{32}P in RNA molecules was verified by enzymatic digestion of an RNA sample from each time point depicted in Fig. 1 and 2 and analysis of digestion products. Figure 4 shows the time-dependent increase of ^{32}P incorporation in internal phosphodiester bonds (peak 2; 3'-monophosphates) as compared with the putative RNA polynucleotide kinase products (peak 4; 5',3'-diphosphates). These data are quantitatively summarized in Fig. 5, which shows the transient increase in 5'-monophosphate with continued increase in internal phosphate groups. These results agree with the alkaline phosphatase data of Fig. 1 and 2 and strongly suggest that the kinase products of half-tRNA-sized molecules are precursors to the ^{32}P found in the internal phosphodiester bonds of tRNA-sized molecules.

The pooled mononucleotide peak from Fig. 4b and c was further analyzed by two-dimensional thin-layer chromatography, and the ^{32}P distribution between 3'-monophosphates of this peak is shown in Table 2. The distribution of ^{32}P in these 3'-monophosphates was very similar to that obtained from digestion of RNA eluted from tRNA region (Table 2) and also total 12-3S RNA,

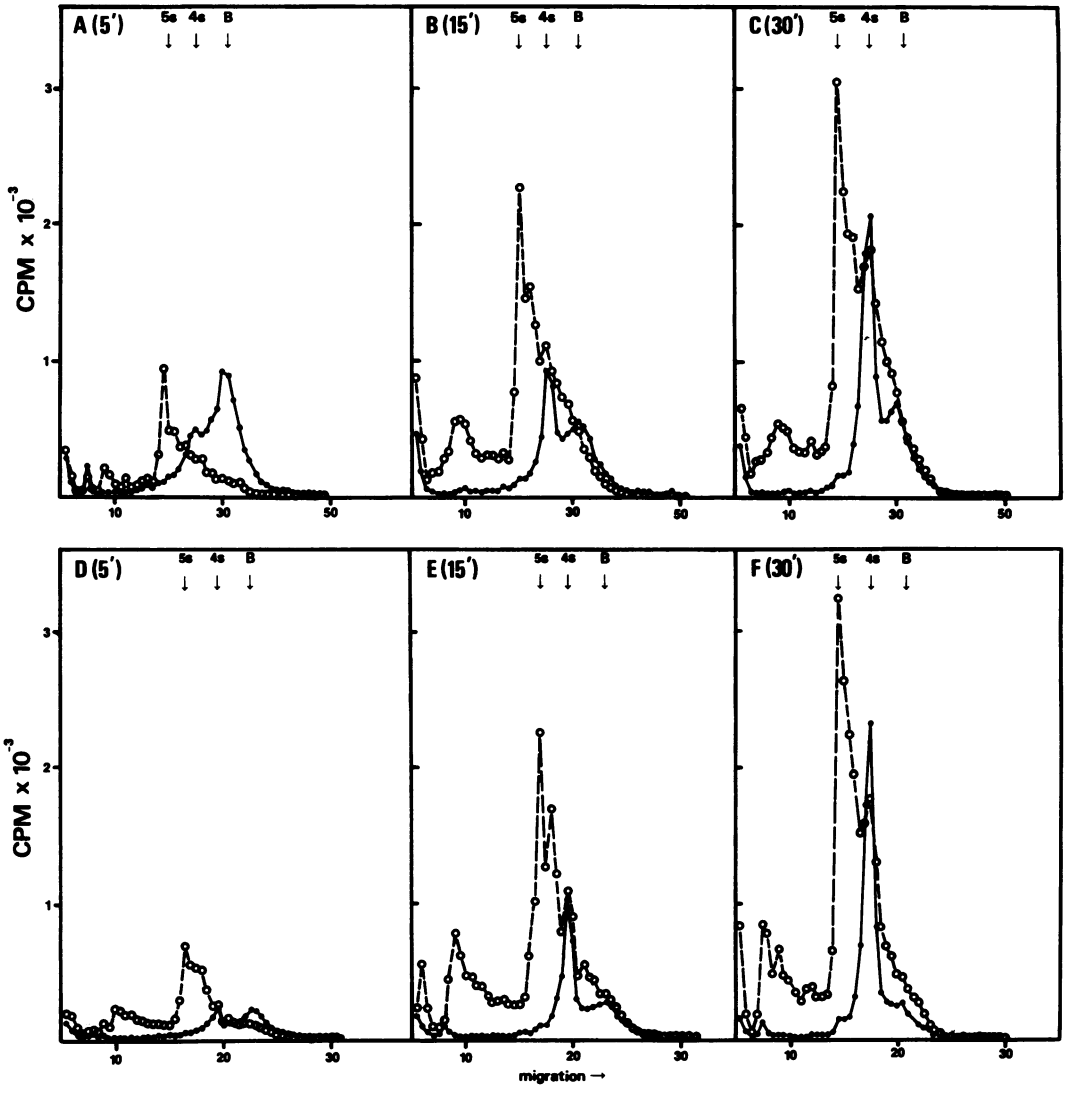

FIG. 2. Time-dependent alkaline phosphatase-insensitive incorporation of γ-^{32}P from ATP in 4S RNA. Duplicate samples of the [γ-^{32}P]ATP- and [^{3}H]UTP-labeled RNA shown in Fig. 1 were treated with 0.4 U of alkaline phosphatase per ml and analyzed by tube gel electrophoresis. Samples and labeling time: (A) control, 5 min; (D) alkaline phosphatase treated, 5 min; (B) control, 15 min; (E) alkaline phosphatase treated, 15 min; (C) control, 30 min; (F) alkaline phosphatase treated, 30 min. Each fraction represents a 2-by-1.1-mm gel slice. Radioactive counts per fraction: (○) [^{3}H]UMP; (●) ^{32}P from [γ-^{32}P]ATP.

indicating that most of the internally incorporated ^{32}P counts in this size fraction are localized in tRNA-sized molecules.

Since we are dealing with total L-cell tRNA population and observed a limited number of bands labeled with ^{32}P in the 32- to 45-nucleotide region, the oligonucleotides containing ^{32}P in internal phosphodiester bonds, given ligation, should also be limited in number as compared to a total T_1 digest of eluted tRNA bands labeled with [α-^{32}P]ATP. Even though the separation is one dimensional, Fig. 6 shows fewer oligonucleotide bands in the T_1 digest of γ-^{32}P-labeled tRNA than α-^{32}P-labeled tRNA, consistent with the previous data.

^{32}P incorporation from [γ-^{32}P]ATP in vitro in RNA molecules synthesized in vivo. The results in Table 1 had suggested that γ-^{32}P incorporation from ATP was not closely linked with transcription, as shown by relative insensitivity to 100 μg of α-amanitin per ml. The rapid appearance of γ-^{32}P from ATP in 32- to 45-nucleotide-long RNA

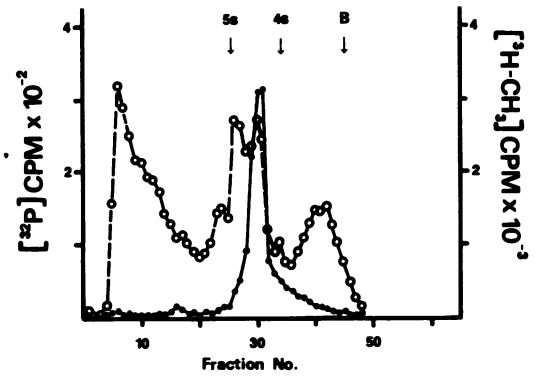

FIG. 3. In vitro synthesis and methylation of L-cell nuclear RNA. RNA synthesis was carried out in the presence of [α-^{32}P]UTP (0.2 mM; specific activity, 308 mCi/mmol) and *S*-[^{3}H]adenosylmethionine (3.6 μM; specific activity, 7.5 Ci/mmol) for 10 min. The gradient-separated 12-3S RNA was analyzed by tube gel electrophoresis as described for Fig. 2. Counts per fraction: (○) ^{32}P; (●) ^{3}H, in—C^{3}H$_3$.

molecules and even tRNA-sized molecules in a 5-min incubation (Fig. 1) suggested the presence of a pool of in vivo-synthesized RNA molecules which could act as acceptors for RNA polynucleotide kinase activity. This is supported by the virtual absence of radioactivity in the 32- to 45-nucleotide region in a 5-min incubation due to RNA synthesis incorporating [α-^{32}P]UTP (Table 1, Fig. 1) or [^{3}H]UTP (Fig. 2A) in the same time period.

Data presented in Fig. 7 further support the presence of a precursor pool for processing. The 32- to 45-nucleotide-long RNA species are labeled with γ-^{32}P from ATP even if 95% of the RNA (lanes a' to c') synthesis is inhibited with 100 μg of α-amanitin per ml as measured by [^{3}H]UMP incorporation. Some γ-^{32}P is also incorporated in internal phosphodiester bonds from ATP in tRNA-sized molecules under these same conditions. Lanes a to c (Fig. 7) show the time course of [α-^{32}P]ATP incorporation for this experiment. Although RNA synthesis has been inhibited by 95% as measured by [^{3}H]UTP incorporation and absence of 5S and pre-tRNA bands in Fig. 7, [α-^{32}P]AMP continues to be incorporated in mature tRNA-sized RNA, presumably by addition of the 3' terminal A of the (3')CCA terminus. It is interesting to note in Fig. 7 (lane e), with overexposure of the tRNA synthesized in the presence of 100 μg of α-amanitin per ml, we can also detect [α-^{32}P]ATP-labeled bands in the 32- to 45-nucleotide region after a 30-min incubation which correspond to the bands labeled with [γ-^{32}P]ATP. Such a finding would be expected if the RNA polynucleotide kinase labeled the 5'-OH end of the 3' half of

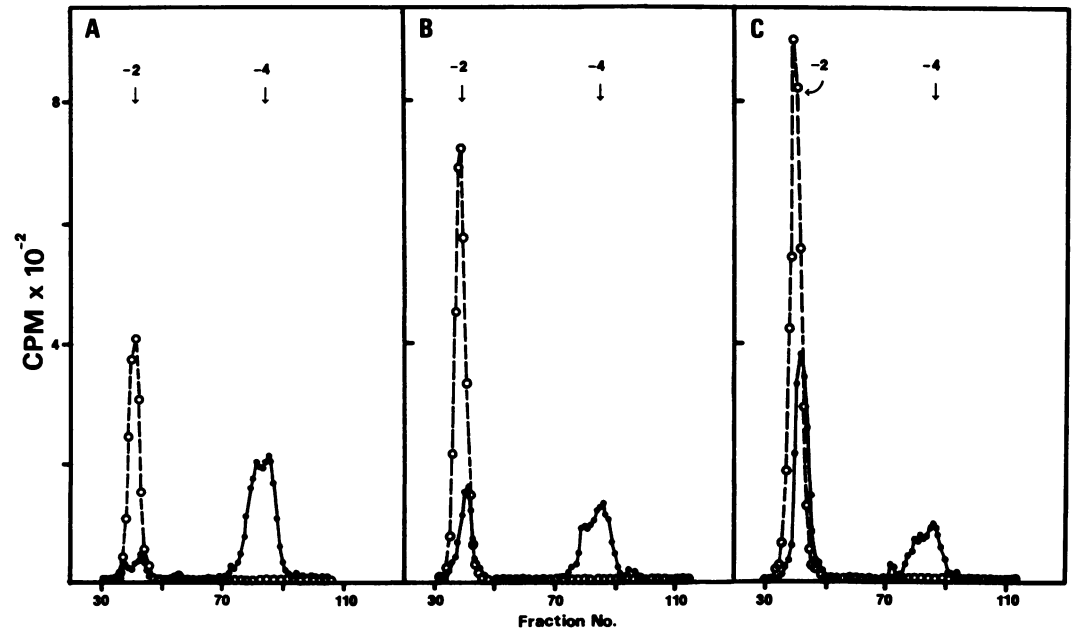

FIG. 4. DEAE-Sephadex chromatography of RNase T$_2$, T$_1$, and A digestion products of RNA synthesized in vitro in the presence of [^{3}H]UTP, [γ-^{32}P]ATP and 1 μg of α-amanitin per ml for 5 (A), 15 (B), or 30 (C) min. Samples of RNA from the experiment shown in Fig. 1 and 2, labeled as above, were digested and analyzed by column chromatography. Digest A in this figure corresponds to RNA in Fig. 2a, digest B corresponds to Fig. 2b, and digest C corresponds to Fig. 2c. Counts per fraction: (○) [^{3}H]UMP; (●) ^{32}P from [γ-^{32}P]ATP.

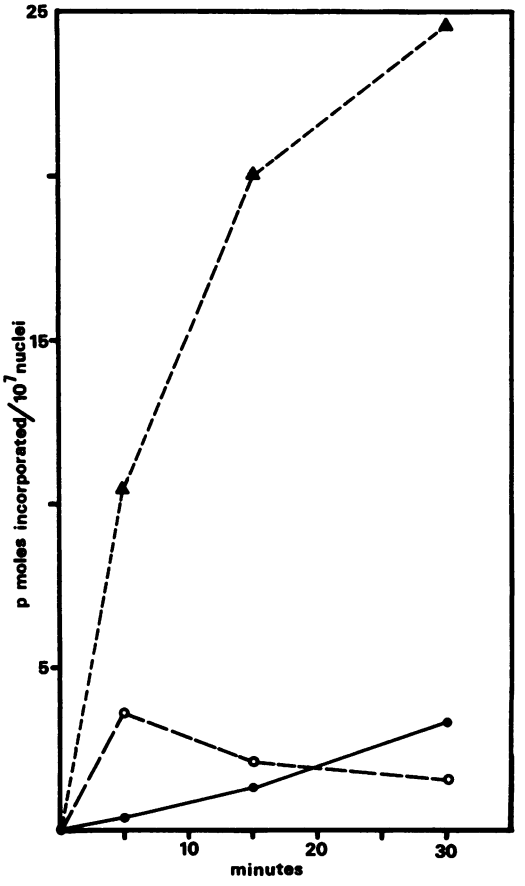

FIG. 5. Summary of RNA synthesis and γ-^{32}P incorporation from ATP in 5'-monophosphoryl termini and internal phosphodiester bonds. Data are summarized from Fig. 4 by sum of counts in each peak and expressed as picomoles per 10^7 nuclei. (▲) [^{3}H]UMP; (○) ^{32}P in ^{32}pXp termini (charge, −4); (●) ^{32}P in internal phosphodiester bonds as measured in 3'Xp (charge, −2).

the tRNA molecules, which already had undergone the CCA modification step at the 3' end of the molecules.

Since these results were obtained in an experiment where the specific activity of [α-^{32}P]ATP was 0.3 Ci/mmol and that of [γ-^{32}P]ATP was 1.1 Ci/mmol, we can rule out the possibility that the internally incorporated γ-^{32}P which appears in the mature tRNA species is due to recycling of the γ-^{32}P from ATP via the α position of ATP. This conclusion is further supported by two-dimensional gel separation of the RNA species eluted from the tRNA region and labeled with [α-^{32}P]UTP (Fig. 8A) or [γ-^{32}P]ATP (Figure 8B). The [α-^{32}P]UTP labels all tRNA species transcribed during the nuclear incubation period. The [γ-^{32}P]ATP should only label RNA species having undergone the proposed ligation and therefore label only a subset of the species labeled with [α-^{32}P]UTP. This appears to be the case, since Fig. 8B shows what appears to be a subset of the pattern observed in Fig. 8A, where several of the spots labeled with [α-^{32}P]UTP cannot be detected with [γ-^{32}P]ATP.

DISCUSSION

Mouse L-cell nuclei can be shown to transcribe endogenous DNA in vitro, resulting in continued accumulation of products of RNA polymerase II (74%) and RNA polymerase III (21%). The predominant species of RNA transcribed by RNA polymerase III are 5S RNA and precursor tRNA, both of which can be completely inhibited by 100 μg of α-amanitin per ml (Fig. 7a, b, and c).

The nuclei also appear to have an active complement of tRNA processing activities as seen from pre-tRNA methylation with *S*-[^{3}H]-adenosylmethionine and the time-dependent processing of pre-tRNA sized molecules to molecules which migrate on gel electrophoresis in position with tRNA bands. The nuclear location of tRNA processing events has been shown by Lönn (14) in *Chironomus* and by Melton et al. (15) in frog oocytes, with recent localization of the tRNA splicing enzymes (5) inside the *Xenopus* oocyte nucleus.

Using the L-cell nuclear system we have been able to show time-dependent incorporation of ^{32}P from [γ-^{32}P]ATP in internal phosphodiester bonds of tRNA-sized molecules. At the same time, pre-tRNA and 5S RNA do not show [γ-^{32}P] incorporation from ATP in internal positions, indicating that the tRNA labeling is due to a posttranscriptional processing event. This event involves a limited set of oligonucleotides as shown by electrophoretic analysis of T_1 digests and nearest-neighbor analysis.

We have also found a number of discrete

TABLE 2. Nearest-neighbor distribution of internal ^{32}P counts in tRNA 3'-nucleotide monophosphates from [γ-^{32}P]ATP[a]

T_2, T_1, and A digest	% cpm				
	Gp	Up	Cp	Ap	P_i
RNA (Fig. 4b and c)	1	4	74	6	15
Eluted tRNA band	6	2	82	10	ND

[a] RNA was digested and separated by two-dimensional thin-layer chromatography. Solvent in first dimension; isobutyric acid–0.5 M NH_4OH (5:3); solvent in second dimension, 5% ammonium acetate, pH 3.5–isopropanol (6:25). The 3'-monophosphate spots were visualized by UV absorbance, scraped, eluted, and counted. Total ^{32}P counts per plate were 340 cpm for the RNA and 418 cpm for the eluted tRNA. ND, Not determined.

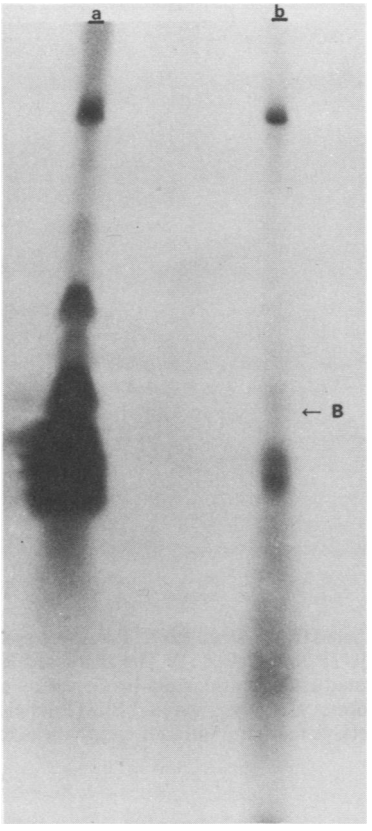

FIG. 6. Separation of T_1 oligonucleotides from 4S RNA labeled in the presence of 1 μg of α-amanitin per ml with [α-^{32}P]ATP or [γ-^{32}P]ATP, by 20% acrylamide-bisacrylamide–urea gel electrophoresis. The extracted RNA was digested with 15 U of T_1 nuclease at 37°C for 3.5 h followed by 0.2 U of alkaline phosphatase per ml for 30 min. The digest was analyzed by slab gel electrophoresis. (a) [α-^{32}P]ATP-labeled RNA; (b) [γ-^{32}P]ATP-labeled RNA. B indicates position of brilliant blue tracking dye.

bands of RNA in the 32- to 45-nucleotide size which become transiently labeled at the 5' termini with γ-^{32}P from ATP presumably by the action of RNA polynucleotide kinase. A number of experimental data suggest that these are 3'-half tRNA molecules in the process of undergoing ligation. (i) They are transcripts of RNA polymerase III as determined by α-amanitin sensitivity; (ii) they are discrete bands of half-tRNA size; (iii) they are the first to become labeled at their 5' termini in our assay with γ-^{32}P from ATP, with a subsequent shift of the majority of the label to the internal phosphodiester bonds of tRNA-sized molecules; (iv) by gel electrophoresis [α-^{32}P]UMP incorporation shows a diffuse area of labeled material in this region with time, whereas [α-^{32}P]AMP incorporation reveals the presence of individual bands with comigrate with those labeled with [γ-^{32}P]ATP. Since the [α-^{32}P]AMP labeling is apparent even in the near absence of RNA synthesis (100 μg of α-amanitin per ml), the data suggest that the discrete bands represent 3'-half tRNA molecules which have undergone CCA 3'-end addition in the isolated nuclei and RNA polynucleotide kinase phosphorylation at the 5' end while in the process of undergoing splicing.

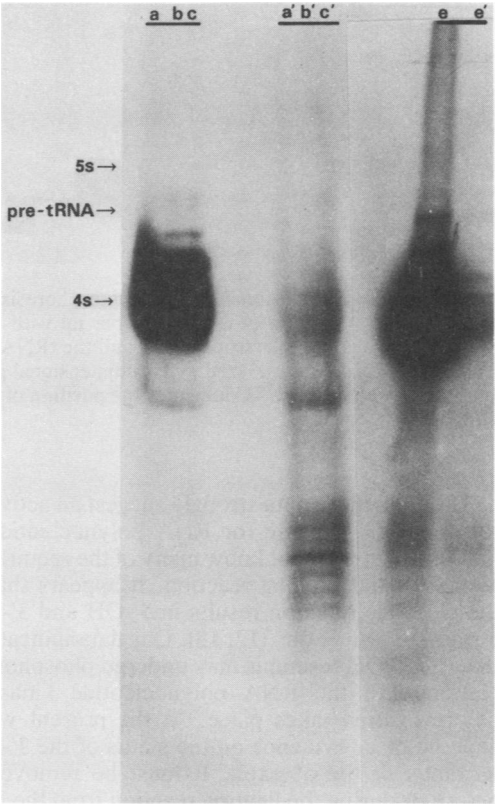

FIG. 7. Time course of accumulation of ^{32}P label in RNA from [α-^{32}P]ATP and [γ-^{32}P]ATP in the presence of 100 μg of α-amanitin per ml. RNA synthesis was carried out in the presence of α-amanitin for 5, 15, or 30 min in the presence of 0.5 mM ATP either as [α-^{32}P]ATP (specific activity, 292 mCi/mmol) or [γ-^{32}P]ATP (specific activity, 1.1 Ci/mmol) and 0.3 mM [^{3}H]UTP (specific activity, 625 mCi/mmol). [^{3}H]UMP incorporation showed a 95% inhibition of RNA synthesis with respect to controls for this experiment. Lanes a, b, and c show RNA labeled for 5, 15, and 30 min with [α-^{32}P]ATP; lanes a', b', and c' show the same time course of incorporation of ^{32}P from [γ-^{32}P]ATP. Lane e shows a longer exposure from another gel of 30-min [α-^{32}P]ATP-labeled RNA in the presence of 100 μg of α-amanitin per ml, with distinct bands in the 32- to 45-nucleotide range; e' shows 30-min [γ-^{32}P]ATP-labeled RNA.

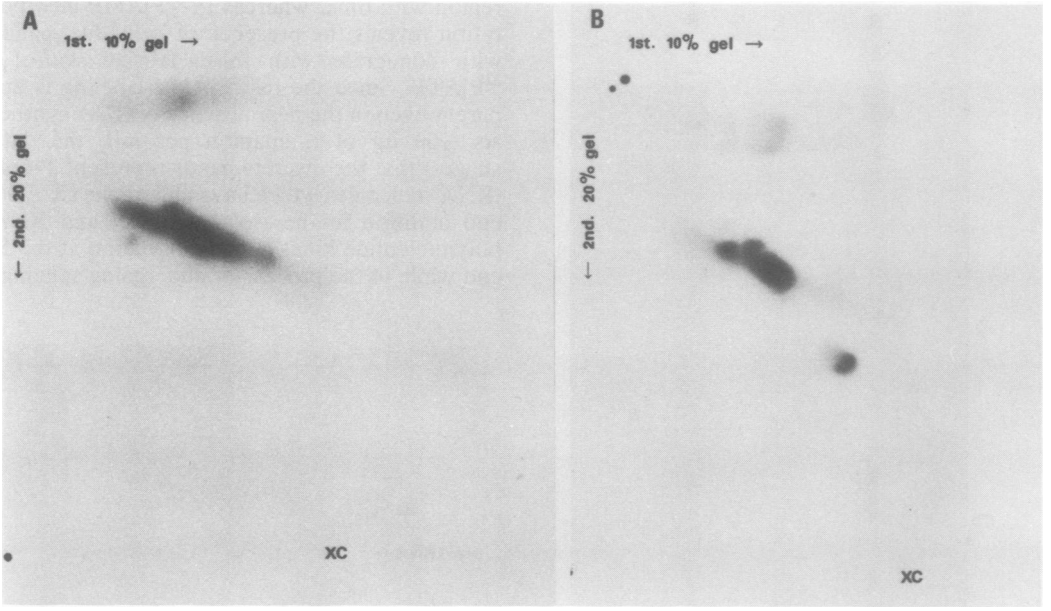

FIG. 8. Two-dimensional slab gel electrophoresis of in vitro-labeled tRNA-sized RNA. RNA was synthesized in the presence of 1 μg of α-amanitin per ml with either [α-^{32}P]UTP or [γ-^{32}P]ATP. The extracted RNA was separated by disk gel electrophoresis, and the tRNA band was eluted after identification by Cerenkov counting. The eluted RNA was analyzed by two-dimensional gel electrophoresis as shown. (A) [α-^{32}P]UTP-labeled RNA; (B) γ-^{32}P-labeled RNA. XC indicates the position of xylene cyanol dye marker. Autoradiography was for 7 days at room temperature.

Although these data strongly suggest an active role in RNA splicing for RNA polynucleotide kinase, we still do not know many of the requirements for the splicing reaction. It appears that the cleavage reaction results in 5′-OH and 3′-P termini of two exons (12, 18). Our data indicate that the 5′-OH terminus may undergo phosphorylation with the RNA polynucleotide kinase before ligation takes place. At the present we have no clear evidence on the status of the 3′-P terminus of the 5′ exon. It must be removed before or during the ligation reaction from the 3′ position, assuming that the RNA polynucleotide kinase-labeled product becomes joined in the internal phosphodiester bond. Konarska et al. (13) have shown that a wheat germ RNA ligase is capable of catalyzing a 3′,5′-phosphodiester bond formation in the presence of a 2′-phosphomonester. This type of mechanism would suggest that the 3′-phosphate is transferred to the 2′ position before removal from the ligated molecule. Our data are consistent with this model, which requires a phosphate group, different from the 3′-phosphate, for the 3′,5′-phosphodiester bond formation.

The ligation reaction is dependent on ATP in the yeast system (12, 16, 19), but does not show such a requirement for rRNA splicing in *Tetrahymena* nucleoli (3). L-cell nuclei require high concentrations of ATP for both the RNA polynucleotide kinase and subsequent internal incorporation of the kinase products in internal phosphodiester bonds by ligation (Winicov, unpublished data). We have found variations in the level and ratio of accumulated RNA polynucleotide kinase products and the ligated ^{32}P-labeled products which strongly correlate with nuclear concentration in the incubation system and levels of ATP which can be maintained during the course of incubation (Winicov, unpublished data).

Since we are dealing with the unfractionated tRNA population of mouse cells, it is difficult to determine the processing order, such as shown for yeast tRNA^{tyr} in *Xenopus* oocytes by Melton et al. (15). There are, however, several observations which can be made from the data presented in this paper. (i) There is a sizable pool of tRNA precursor molecules which continue to undergo processing when RNA transcription is inhibited by 100 μg of α-amanitin per ml. (ii) Many of the methylations in these nuclei take place at the tRNA precursor level. (iii) Only a fraction of the tRNA precursor molecules synthesized in vitro also appear to be processed in vitro to the mature tRNA size as seen in Fig. 1. (iv) The terminal A addition in (3′) CCA appears to occur late in tRNA processing as shown by Melton et

al. (15), since the mature tRNA-sized molecules continue to be labeled in the absence of RNA synthesis. We cannot rule out at this time the possibility that the labeling observed with [α-^{32}P]ATP in the absence of RNA synthesis is due to an exchange reaction at the 3' terminus (7). It has been suggested for the frog oocyte system (15) that splicing of tRNA species may be the last processing event, even though in vitro the cleavage enzyme "XlaI" and ligation do not seem to require removal of the 5' leader and 3' trailer sequences.

The data presented in this paper strongly suggest that several mouse L-cell tRNA species undergo splicing in isolated nuclei. The RNA polynucleotide kinase appears to take an active part in the splicing sequence, and the phosphorylated 3'-half tRNA molecules become ligated to yield mature tRNA-sized molecules. These results suggest that the ligation mechanism must involve a 3'-OH and 5'-P group for the formation of the new phosphodiester bond.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Kim L. Cairney and helpful discussions with Jeffrey Ross and Craig Peebles.

This research was supported by a National Science Foundation grant (79 22207) and by Public Health Service grant GM-27230 from the National Institutes of Health to I.W.

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