ABSENCE OF DETECTABLE RNA LIGASE ACTIVITY IN EUKARYOTIC CELLS

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SUMMARY: Reports of the existence of eukaryotic RNA ligases may be incorrect. Evidence for this activity has been based upon the conversion of [5'-^{32}P]-terminated oligoribonucleotides to an alkaline phosphatase resistant form and upon the detection of radioactive ribonucleoside monophosphates after alkaline hydrolysis of the reaction products. Although we have in part confirmed these observations, we find the labeled ribonucleoside monophosphate to be the 5'-isomer, and not the expected 2'(3')-isomer. In addition, roughly equivalent amounts of ribonucleoside monophosphate were observed whether or not alkaline hydrolysis was performed. We conclude that the existence of RNA ligase activity in eukaryotic cells is suspect.

INTRODUCTION: RNA ligase was discovered in T4-infected E. coli and has been shown to catalyze the intra- and intermolecular formation of phosphodiester bonds with various RNA substrates (1-7). It has been assayed by measuring the formation of alkaline phosphatase resistant material from [5'-^{32}P]-terminated oligoribonucleotides and by the formation of labeled ribonucleoside monophosphates (NMPs) following alkaline hydrolysis of the reaction products.

The above assays have been used in the search for possible eukaryotic RNA ligase activities (2,5,7). Using these procedures, we have found similar amounts of NMPs whether or not alkaline hydrolysis was performed. In addition, the NMPs detected were almost exclusively the 5'-isomers. We have been unable to detect RNA ligase activity in several different eukaryotic cell lines, and we feel that the existence of RNA ligase activity in eukaryotic cells is in question.

MATERIALS AND METHODS: Poly(I) of average chain length 65 (P-L Laboratories) was converted to the [5'-^{32}P]-derivative as described previously (3). Reaction mixtures were incubated at 37^{o} and were assayed for alkaline phosphatase (BAP C,

Worthington) resistance, and/or NMP formation by adsorbing and eluting nucleotides from charcoal before and after alkaline hydrolysis (3). Nucleotides were separated by high voltage electrophoresis (8), eluted and chromatographed in a solution containing 6 parts [M ammonium acetate and 3.3 mM EDTA saturated with boric acid (adjusted to pH 7.0 with concentrated NH_4OH)] and 4 parts [absolute ethanol] (v/v) on cellulose coated thin layer plates (Eastman), to separate 2'(3')-NMPs from the 5'-isomers.

RESULTS: With eukaryotic cell extracts, greater than 90% of the counts which co-migrated with the 2'(3')-IMP marker on electrophoresis at pH 4.0, were subsequently recovered in the 5'-IMP region when the isomers were separated by chromatography (Table I). At pH 4.0 both the 2'(3')- and 5'-isomers of IMP, AMP and CMP co-migrate during electrophoresis (data not shown). In addition, the small amounts of 2'(3')-IMP detected in some experiments were not increased by alkaline hydrolysis (Table I). This finding is inconsistent with the formation of a phosphodiester linkage between a [5'-^{32}P]phosphate and a 3'-hydroxyl

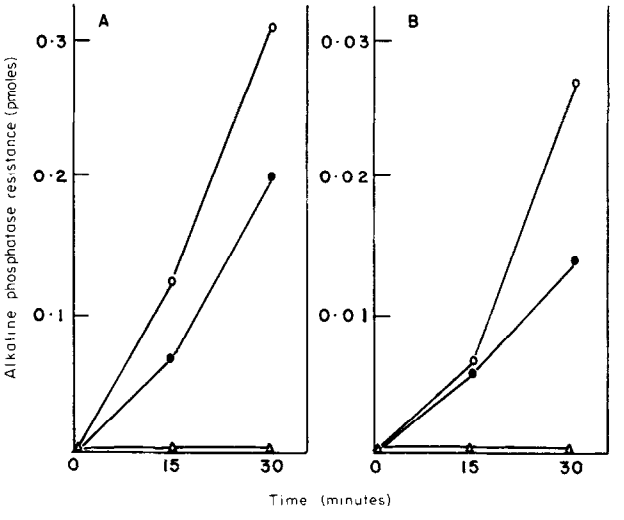

Figure 1. Effect of added L cell extract on T4 RNA ligase activity. T4 RNA ligase was added to a 50 µl reaction in the presence (●) and in the absence (o) of 0.4 mg/ml of L cell extract, or L cell extract was assayed alone (Δ). All were assayed as described in materials and methods. In A, 0.65 units of T4 RNA ligase were added to each reaction, and in B, 0.06 units of enzyme were added. The pmoles of [^{32}P] rendered insensitive to alkaline phosphatase are plotted as a function of reaction time. A zero time background of 0.007 pmoles was subtracted from each value. The specific activity of the [5'-^{32}P]poly(I) used was 2.95 x 10^4 cpm/pmole and 0.85 pmoles were added to each reaction.

Table I. Distribution of Nucleoside Monophosphates Following Ligase Assays

Enzyme source	L cell[1]		L cell[2]		JLS-V9[3]		T4[1]	
Alkaline hydrolysis	-	+	-	+	-	+	-	+
2'(3')-NMP (pmoles)	0.13	0.07	<0.01	<0.01	<0.01	<0.01	0.04	0.41
5'-NMP (pmoles)	1.39	1.61	0.06	0.06	0.21	0.18	<0.01	<0.01

Isomers of nucleoside monophosphate formed in the RNA ligase reaction were separated as described in materials and methods.

[1] L cell extracts and T4 RNA ligase were assayed according to Walker et al. (3). [5'-^{32}P]poly(I) with a specific activity of 1.7×10^4 cpm/pmole was used as substrate and 3.52 pmoles were added to each 100 µl reaction.

[2] L cell extracts were assayed according to Linné et al. (5). [5'-^{32}P]poly(I)·poly(C) with a specific activity of 5.9×10^3 cpm/pmole was used as substrate and 6.78 pmoles were added to each 100 µl reaction.

[3] JLS-V9 cell extracts were assayed according to Cranston et al. (7). [5'-^{32}P]poly(C) with a specific activity of 1.5×10^3 cpm/pmole was used as substrate and 16.67 pmoles were added to each 100 µl reaction.

Table II. Effect of Single-stranded DNA and Alkaline Hydrolysis on IMP Formation

	L cell				L cell + T4				T4			
	-DNA		+DNA		-DNA		+DNA		-DNA		+DNA	
Alkaline hydrolysis	-	+	-	+	-	+	-	+	-	+	-	+
2'(3')-IMP (pmoles)	<.01	<.01	<.01	<.01	<.01	.12	.01	.21	.01	.24	<.01	.35
5'-IMP (pmoles)	.59	.85	.35	.28	.60	.57	.23	.19	<.01	<.01	<.01	<.01

L cell protein (25 µg), 0.65 units of T4 RNA ligase or both were assayed in a final reaction volume of 100 µl, and nucleoside monophosphate isomer distribution following adsorption and elution from charcoal is shown. Samples were assayed at 30 minutes in the presence or absence of 25 µg single-stranded calf thymus DNA, both before and after alkaline hydrolysis (0.3 N KOH, 37° for 18 hours). [5'-^{32}P]poly(I) with a specific activity of 2.95 x 10^4 cpm/pmole was used as substrate and 1.69 pmoles were added to each 100 µl reaction.

group. Previous reports on the existence of a eukaryotic RNA ligase activity did not consider the formation of NMPs prior to alkaline hydrolysis nor establish the nature of the isomer formed (5,7). Table I also shows that the purified T4 RNA ligase (3) assayed as a control, does convert [5'-^{32}P](I)$_{65}$ to a product which yields only [2'(3')-^{32}P]IMP after alkaline hydrolysis.

To determine if RNA ligase activity could be detected in eukaryotic cell extracts, we performed a number of mixing experiments with purified T4 RNA ligase and L cell extracts. Amounts of L cell protein identical to those of typical assays (20 µg per 50 µl reaction) were used. At levels of T4 RNA ligase ranging from 0.06 to 0.65 units of enzyme (3), the formation of both alkaline phosphatase resistant counts (Figure 1) and of 2'(3')-IMP from [5'-^{32}P](I)$_{65}$ following alkaline hydrolysis (Table II) was inhibited a maximum of 50% by L cell extracts. However, T4 RNA ligase activity was clearly detectable even at the lowest levels used (Figure 1B). In this experiment, no alkaline phosphatase resistant products were formed by the L cell extract alone.

It is apparent (Table II) that L cell extracts possess a nuclease activity which converts [5'-^{32}P]poly(I) to [5'-^{32}P]IMP. We have found that single-stranded DNA inhibits this nuclease activity, and that it is a more effective inhibitor than equivalent nucleotide concentrations of either yeast RNA or double-stranded DNA (data not shown). In the presence of denatured calf thymus DNA we consistently observed a decrease in the amount of 5'-IMP formed (Table II). The formation of 2'(3')-IMP by the purified T4 RNA ligase, both in the presence and absence of the L cell extract, was not reproducibly affected by the DNA although enhancement is shown in the experiment in Table II.

Linné et al. (5) reported that the specific activity of RNA ligase in L cell extracts was comparable to that found in T4-infected E. coli. Using extracts of L cells and the assay procedures of Linné et al. (5), which include ethanol precipitation prior to alkaline hydrolysis, only small amounts of [^{32}P]IMP were recovered (Table I). It is apparent that ^{32}P was recovered primarily as

5'-IMP, with similar amounts formed both before and after alkaline hydrolysis (Table I). Similarly, applying the assay procedures of Cranston et al. (7) to extracts of JLS-V9 cells in the presence of [5'-^{32}P]poly(C) (provided by R. Silber), resulted in the almost exclusive formation of [5'-^{32}P]CMP (Table I). The formation of this compound was also independent of KOH treatment. We wish to point out that in our experiments, the formation of alkaline phosphatase resistant product was only 0-15% of that reported by others (5,7), and that these levels were highly variable. Substances interfering with alkaline phosphatase action have been found in both the cytoplasmic and nuclear fraction of L cells (5).

On the basis of alkaline phosphatase resistance, we could find little or no detectable RNA ligase activity in extracts prepared from each of the following systems: L cells, reovirus infected L cells at 6 and 14 hours post-infection, JLS-V6, JLS-V9, HeLa, MOP C myoloma cells, or in five different strains of mycoplasma (M. pneumoniae FH, M. hominis, M. arthritidis, M. gallisepticum and M. fermentans K10, gifts of M. Gabridge). In addition, there was no detectable formation of 2'(3')-NMP in any of the above systems with the exception of HeLa and MOP C cells which were not tested. The following modifications had no effect on either the formation of alkaline phosphatase resistant counts or of 2'(3')-NMPs with L cell extracts: addition of S-adenosyl methionine, NAD, CTP, GTP and UTP as possible cofactors to assay mixtures; Dounce homogenization or sonic disruption; dialysis against and assay of cell extracts with Tris·HCl, N-2-hydroxyethylpiperazine-N-2'-ethane-sulfonic acid and phosphate buffers (all at pH 7.5); varying protein concentration from 0.15 to 1.50 mg/ml and substrate concentrations from 5 pmoles to 24 nmoles in 5'-ends/ml per assay; assay of cytoplasmic and nuclear fractions alone; assay of supernatant and sedimenting fractions of 1000, 5000, 10,000, 25,000, and 100,000 x g centrifugations on total L cell lysates; use of [5'-^{32}P]-labeled poly(I), poly(C), poly(I)·poly(C) and poly(A) as substrates.

DISCUSSION: Cranston et al. (7) have been unable to demonstrate an ATP or NAD

requirement for their putative JLS-V9 RNA ligase activity, although they were able to show an ATP requirement for the T4 RNA ligase in the presence of JLS-V9 extracts. In addition, R. Silber has informed us that further experiments with extracts from both JLS-V9 cells and different hepatomas have shown that alkaline hydrolysis of [5'-^{32}P]poly(C) containing reaction mixtures yields NMPs that are completely sensitive to 5'-nucleotidase. In contrast, the NMPs derived from the T4 RNA ligase product are insensitive to 5'-nucleotidase (R. Silber, personal communication.) The small amounts of 2'(3')-NMP formed during some of our assays may have been due to a phosphotransferase activity, since its production was alkaline hydrolysis independent.

Because the NMPs formed as products in our RNA ligase assays are the 5'- rather than the 2'(3')-isomers, because there is no requirement for alkaline hydrolysis for NMP production, and because we have not been able to demonstrate RNA ligase activity in any of the cell lines tested, including those in which it has been reported, we conclude that the existence of a eukaryotic RNA ligase activity is in doubt.

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