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IDENTIFICATION OF RIBONUCLEASE P ACTIVITY FROM CHICK EMBRYOS

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Summary

RNAase P (EC 3.1.26.5) activity has been identified in chick embryo thigh tissue on the basis of specific cleavage of *Escherichia coli* 129 nucleotide tRNA^{Tyr} precursor and has been partially purified by the procedure used for human tissue culture KB cell RNAase P. RNAase P from chick resembles the KB cell RNAase P in substrate specificity, requirement for a divalent cation (Mg^{2+}) and a monovalent cation (K^+, Na^+ or NH_4^+) for activity, inhibition by bulk tRNA, ready inactivation by proteases, and increasing instability with purification. RNAase P activity is also present in whole chick embryos, as well as in liver and heart tissues. Furthermore, crude preparations of RNAase P from chick embryo heart tissue are relatively free of contaminating nucleases.

Introduction

The biosynthesis of transfer RNA in both eucaryotes and procaryotes requires the action of nucleolytic and nucleotide modification enzymes. In *Escherichia coli*, one such enzyme is RNAase P (EC 3.1.26.5), an endonuclease that catalyzes the release of extra nucleotides from the 5′-end of precursor tRNA molecules to yield the 5′-terminus of the corresponding mature tRNA sequence [1]. In eucaryotes, RNAase P activity has been identified in human tissue culture KB cells [2], silkworm extracts [3] and tissue cultured monkey kidney cells [4]. Partial purification and characterization of RNAase P from KB cells showed that the eucaryotic and procaryotic enzymes are similar in

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substrate specificity, ion requirements, sensitivity to nuclease inhibitors, and inactivation by micrococcal nuclease [1,2,5—7]. Two features of *E. coli* RNAase P, its buoyant density and sensitivity to micrococcal nuclease, indicate that it is made of both RNA and protein components [6,7]. Comparative structural analysis of RNAase P from KB cells and *E. coli* has not yet been feasible due to low yields and instability of the eucaryotic enzyme during purification [5]. Clearly, there is a need for a readily available eucaryotic source of large amounts of RNAase P.

In this report, we describe the partial purification and characterization of RNAase P from chick embryo thigh tissue and show its close similarity to the enzyme from human KB cells. We also identify RNAase P activity in whole chick embryos and in extracts from chick embryo heart and liver tissues. Since crude preparations from chick embryo heart tissue show very little contaminating nuclease activity, heart tissue from a large animal may be a good source of RNAase P.

Methods

Partial purification of RNAase P from chick embryo thigh tissue. Thigh tissues were dissected by hand from 12—48 chick embryos on the 12th, 13th or 14th day of development. The thigh tissues were suspended in homogenization medium (12 thighs/8 ml) containing 25 mM KCl, 10 mM $MgCl_2$, 25 mM sucrose, 0.1 mM β-mercaptoethanol, and 10 mM Tris/HCl buffer (pH 7.6), and disrupted with at least five passes in a Dounce homogenizer. Heart and liver tissues were treated similarly. Whole chick embryos, minus eyes and beaks, were disrupted in a Waring Blendor. The homogenate was centrifuged at $750 \times g$ for 5 min, the pellet was then suspended and rehomogenized in homogenization medium (half the original volume) and centrifuged at $3000 \times g$ for 5 min. The resultant supernatant was then centrifuged at $20\,000 \times g$ for 20 min, yielding the $20\,000 \times g$ supernatant. Subsequent purification followed the procedure used successfully to isolate RNAase P from human KB cells [2]. The $20\,000 \times g$ supernatant was fractionated with ammonium sulfate and activity was found in the 10—25% (0.1 to 0.25 g/ml original volume of $20\,000 \times g$ supernatant). Following suspension in and dialysis against buffer B (20 mM NH_4Cl, 10 mM magnesium acetate, 6 mM β-mercaptoethanol, and 20 mM Tris-HCl, pH 7.6), the 10—25% ammonium sulfate fraction was further purified on a DEAE-Sephadex A-50 column [2]. Activity was eluted when the NH_4Cl concentration in buffer B was raised to 0.3 M; 5% sucrose was added to the eluants.

RNAase P assays. The standard assay conditions for enzymatic cleavage of tRNA precursors were as described previously [2] except that the reaction mixture routinely contained 100 mM KCl in place of 100 mM NH_4Cl; the precursor to *E. coli* $tRNA^{Tyr}$ was used as substrate. For determinations of ionic requirements, RNAase P eluants from the DEAE-Sephadex A-50 column were dialyzed against a low salt buffer (5 mM NH_4Cl, 0.1 mM magnesium acetate, 6 mM β-mercaptoethanol, 5% sucrose, and 5 mM Tris/HCl, pH 7.6) prior to the activity assay.

Protease digestion of RNAase P. The standard protease assay contained 50

μg protease and chick embryo thigh tissue extract, containing RNAase P in buffer B with 0.3 M NH_4Cl and 5% sucrose in a final volume of 35 μl. The mixture was incubated at 37°C for 30 min and then placed in ice. RNAase P activity in 10 or 20 μl aliquots was promptly measured as usual.

Protein determinations. Proteins were assayed by the methods of Lowry et al. [8] or of Bradford [9] with bovine serum albumin as standard.

Materials. Eggs, fertilized 5—7 days previously, were purchased from Spafas, Inc. (Norwich, CT) and incubated at 37°C until used for isolation of thigh tissue. Bacterial and bacteriophage strains were the same as used previously [1—2]. *E. coli* RNAase P was purified according to the method of Robertson et al. [1]. Subtilisin BPN′ was obtained from Sigma. The sources of other enzymes and chemicals, and the preparations of chromatographic media and radioactive polynucleotides were as described previously [2].

Results

RNAase P activity in chick embryo thigh tissue

Chick embryo thigh tissue was assayed for RNAase P activity by incubating tissue extracts with a ^{32}P-labeled *E. coli* $tRNA^{Tyr}$ precursor. The reaction mixture was analyzed by polyacrylamide gel electrophoresis and autoradiography to separate and visualize the reaction products. A $tRNA^{Tyr}$ precursor-cleaving activity can be detected in the 20 000 × *g* supernatant (Fig. 1). Comparison of the reaction products of the crude activity found in chick embryo extracts (lane 3) with those made by *E. coli* RNAase P (lane 2) reveals considerable contamination by degradative nucleases and by 3′-exonuclease 'trimming' activity. Ammonium sulfate fractionation of the 20 000 × *g* supernatant separates the RNAase P-like activity from other nuclease activity as shown by the increased recovery of the 3′-fragment of the cleavage reaction (lane 4); however, degradation of the 5′-fragment and 3′-exonuclease 'trimming' activity remain. More highly purified $tRNA^{Tyr}$ precursor-cleaving activity is obtained in the 0.3 M NH_4Cl eluant from a DEAE-Sephadex A-50 column. The active material eluting from the DEAE-Sephadex column is apparently free of contaminating ribonucleases as shown by the recovery of intact 5′- and 3′-cleavage products of the 129 nucleotide $tRNA^{Tyr}$ precursor (lanes 10—13). Note that products of radioautolysis, present in lanes 10—13, are also seen in lane 1 in which no enzyme is added to the reaction mixture. RNAase A fingerprint analysis of the cleavage products (Fig. 2) of the $tRNA^{Tyr}$ precursor is identical to previously published fingerprints of RNAase P cleavage products and verifies that the cleavage site is identical to that obtained with KB cell and *E. coli* RNAase P [1,2].

A purification factor of specific RNAase P activity from chick embryo tissue as measured by tRNA precursor cleavage cannot be determined due to the presence of contaminating ribonucleases in cruder fractions. On the basis of protein recovery, the activity was purified approximately 60-fold from the 20 000 × *g* supernatant. For further purification, preliminary experiments indicate that the gel electrophoresis procedures used for *E. coli* RNAase P [6] could be useful.

Chick RNAase P in more highly purified fractions is less stable than the

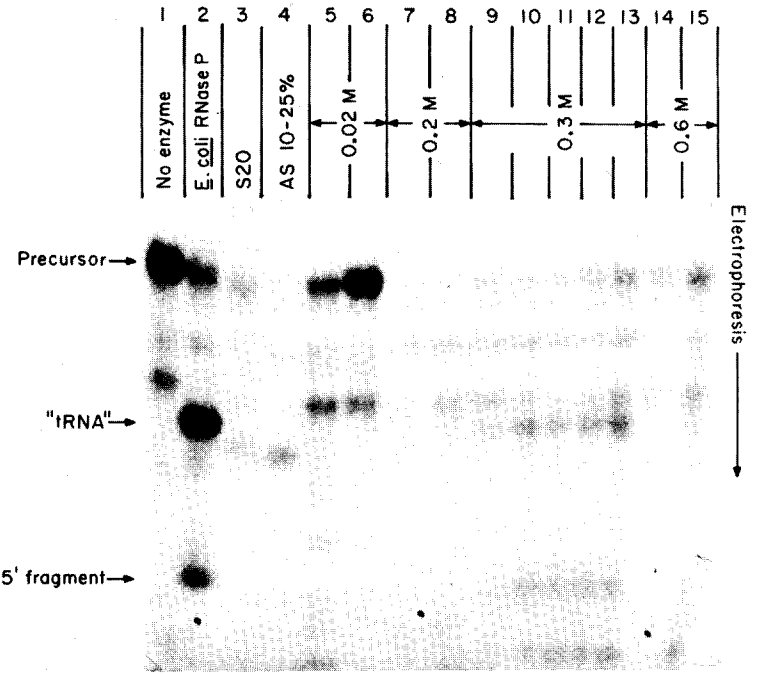

Fig. 1. Partial purification of chick embryo thigh RNAase P. Thigh tissues from 14-day-old eggs were homogenized and fractionated. Aliquots of each fraction were saved for determination of RNAase P activity by the standard radionucleotide assay. Lane 3 represents the 20 000 × *g* supernatant, lane 4 contains the 10—25% (w/v) ammonium sulfate fraction, and lanes 5—15 represent fractions (initial ones for each step) eluted from a DEAE-Sephadex A-50 column with a stepwise salt gradient formed from buffer B containing 0.02 M, 0.2 M, 0.3 M and 0.6 M NH_4Cl. 10 μl (20 000 × *g* supernatant and $(NH_4)_2SO_4$ fractions) or 40 μl (column eluants) of enzyme and 8000 cpm of ^{32}P-labeled $tRNA^{Tyr}$ precursor (129 nucleotides) were used in each reaction. Products of radioautolysis (no enzyme added) are apparent in lane 1. 'tRNA' and 5'-fragment refer to the RNAase P cleavage products of the precursor to *E. coli* $tRNA^{Tyr}$ containing, respectively, the mature tRNA sequence and the 'extra' 41 nucleotides at the 5'-end of the precursor.

activity found in cruder fractions. While good activity can be recovered from a 10—25% (w/v) ammonium sulfate preparation stored at 4°C for up to 2 months, column purified enzyme loses activity in 1 or 2 weeks. Column-purified enzyme can be concentrated by precipitation with 0.5 g of $(NH_4)_2SO_4$ per ml, suspended in a small volume of buffer, and stored in liquid nitrogen with little loss in activity. As RNAase P activity is lost in column purified enzyme preparations, residual contamination by degradative nucleases is detected in the standard radionucleotide activity assay. These ribonucleases may be a contributory cause of instability [5].

Substrate specificity

The RNAase P from chick embryo thigh tissue was purified on the basis of its ability to cleave the 129 nucleotide *E. coli* $tRNA^{Tyr}$ precursor. Further evidence that the chick embryo enzyme is like *E. coli* RNAase P is shown by its ability to cleave other *E. coli* precursor RNA molecules to products which comigrate on acrylamide gels with those produced by the E. coli enzyme. Substrates tested include the $tRNA^{Tyr}$ precursor containing only 96 nucleotides

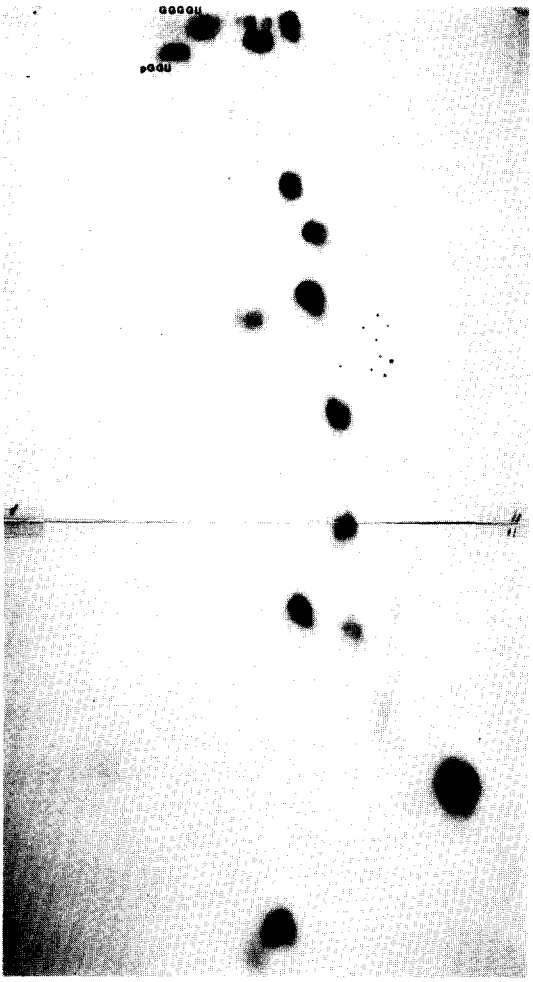

Fig. 2. Products of ribonuclease A digestion of the 'tRNA' fragment produced by cleavage of the 129 nucleotide $tRNA^{Tyr}$ precursor. The cleavage reaction contained 40 µl of chick embryo thigh RNAase P eluted from a DEAE-Sephadex A-50 column by buffer B containing 0.3 M NH_4CL and 500 000 cpm of ^{32}P-labeled precursor. The two cleavage products were separated and extracted as for the preparation of substrate. Recovery was 25 000 cpm of the tRNA fragment and 18 000 cpm of the 5′-fragment for fingerprinting. Ribonuclease A digestion products were separated by electrophoresis on cellulose acetate, 7 M urea (pH 3.5) from right to left, and on DEAE-cellulose paper in 7% formic acid (v/v) from top to bottom.

and a mixture of two precursor RNAs of 180 nucleotides in length [10] (Fig. 3). No cleavage of bacteriophage $\phi 80$ M_3RNA [11] could be detected, but this could be due to the low specific activity of the chick embryo RNAase P preparations.

Factors affected chick RNAase P activity

The effects of ions, tRNA and proteases on chick embryo RNAase P activity were measured by quantitating reaction products separated in acrylamide gels [2]. The data are summarized in the following statements. RNAase P from

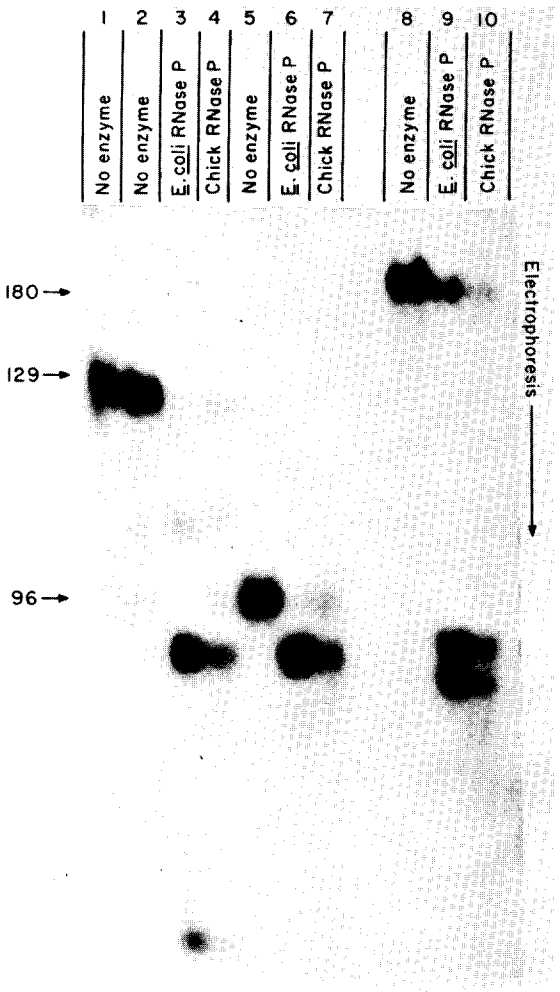

Fig. 3. Cleavage of *E. coli* RNA precursor molecules by RNAase P from *E. coli* and embryonic chick thigh tissues. ^{32}P-labeled precursor RNA species were prepared from *E. coli* A49 as described previously [2]. Cleavage was performed by the standard RNAase P assay. The number of nucleotides in each precursor RNA is 129 for lanes 1—4, 96 for lanes 5—7, and 180 for lanes 8—10. Reactions for lanes 1, 2, 5, and 8 contained no enzyme; for lanes 3, 6 and 9, 5 μl *E. coli* RNAase P; and for lanes 4, 7 and 10, 5 μl of chick embryo thigh RNAase P concentrated by $(NH_4)_2SO_4$ precipitation. 8000 cpm of precursor were used in each reaction.

chick embryo thigh tissue requires Mg^{2+} (Mn^{2+} or Ca^{2+} will not substitute as divalent cations) and a monovalent cation (K^+, Na^+ or NH_4^+) for activity. K^+ gives somewhat better stimulation of activity than Na^+ or NH_4^+. Optimal ion concentrations are approx. 5 mM $MgCl_2$ and 100 mM KCl. High concentrations (400 mM) of either K^+ or NH_4^+ are inhibitory. It should also be pointed out that 3 μM *E. coli* bulk tRNA inhibits the embryonic thigh tissue activity essentially 100%. RNAase P from chick embryo tissue is completely inactivated by the proteases subtilisin BPN′, proteinase K, and pronase. In contrast, RNAase P from *E. coli* is completely resistant to attack by both subtilisin BPN′ and

proteinase K at concentrations of 2 mg/ml, but can be inactivated by pronase [6].

RNAase P activity in other chick embryo extracts

In addition to chick embryo thigh tissue, we examined RNAase P activity in 20 000 × *g* supernatant and subsequent 10—25% (w/v) $(NH_4)_2SO_4$ fractions from whole chick embryos and embryonic heart and liver tissues (Fig. 4). In all cases, 20 000 × *g* supernatant contain RNAase P activity but exhibit considerable degradative ribonuclease and 3′-exonuclease activity. $(NH_4)_2SO_4$ fractions show cleavage of precursor to a mature tRNA sized molecule which has under-

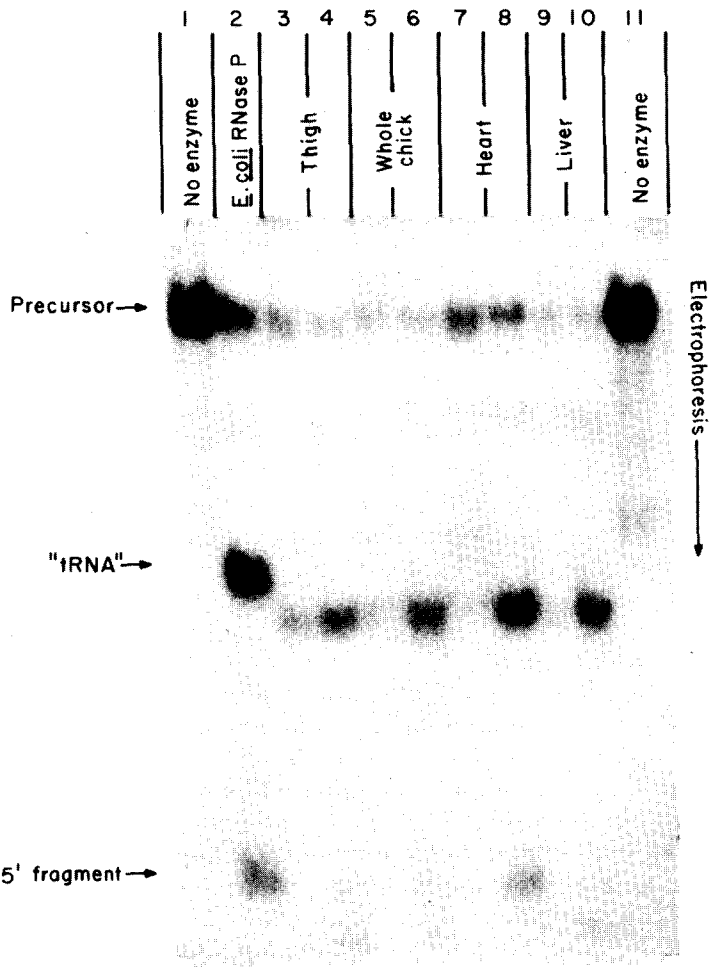

Fig. 4. Identification of RNAase P in several chick embryo extracts. RNAase P activity was measured in 20 000 × *g* supernatant and 10—25% ammonium sulfate fractions prepared from chick embryo thigh tissue (lanes 3 and 4), whole chick embryos (lanes 6 and 7), chick embryo heart tissues (lanes 8 and 9), and chick embryo liver tissue (lanes 10 and 11). Assays for lanes 1 and 11 contained no enzyme. Lane 2 shows *E. coli* RNAase P activity. 10 μl of each 20 000 × *g* supernatant or 5 μl of each $(NH_4)_2SO_4$ fraction and 10^4 cpm of ^{32}P-labeled $tRNA^{Tyr}$ precursor were used in each reaction.

gone trimming at the 3′-terminus. The $(NH_4)_2SO_4$ fraction derived from heart tissue contains far less degradative nuclease contamination than that derived from other tissues. Good recovery of the two RNAase P cleavage products, as well as of uncleaved precursor, is obtained. The 3′-exonuclease 'trimming' activity, however, is still evident.

Discussion

RNAase P activity has been identified in chick embryo tissue on the basis of specific cleavage of *E. coli* $tRNA^{Tyr}$ precursor molecules. The cleavage sites of the chick enzyme are identical to those of *E. coli* and KB cell RNAase P [1—2]. The partially purified chick embryo thigh enzyme is essentially identical in all respects to KB cell RNAase P [2,5]. Both eucaryotic enzymes cleave *E. coli* RNA precursors to products of the same electrophoretic mobility; are partially purified by the same scheme and are increasingly less stable with purification. They both require Mg^{2+} (5 mM optimal) and a monovalent cation (100—200 mM optimal) for activity, are inhibited almost 100% by 3 μM tRNA, and are inactivated by proteases. RNAase P from silkworm glands is similar to the chick and KB cell enzymes with respect to its behavior during purification and its cleavage specificity in *E. coli* tRNA precursors [3]. We have also demonstrated the presence of RNAase P in heart and liver tissues of chick embryos, as well as in whole embryo extracts. Thus, RNAase P clearly can be commonly found and is probably ubiquitous in eucaryotic organisms. Furthermore, all the eucaryotic RNAase P activities which have been examined exhibit very similar characteristics.

RNAase P from chick embryos is similar to *E. coli* RNAase P in substrate specificity, location of cleavage sites, the dependence on Mg^{2+} and a monovalent cation for optimal activity, and its general behavior during purification. However, the chick enzyme differs from the *E. coli* enzyme in several important ways. Chick embryo RNAase P, like the KB cell enzyme [5], elutes from DEAE-Sephadex with the addition of 0.3 M NH_4Cl, becomes less stable during purification, and is readily inactivated by all proteases tried. *E. coli* RNAase P [6] remains bound to DEAE-Sephadex with a 0.4 M NH_4Cl wash and elutes with a 0.5 M NH_4Cl wash, is at least 10-fold more active than the chick of KB cell enzyme at this stage, is quite stable at 4°C for 1 or 2 years in high salt buffer, is resistant to attack by proteases, and is partially protected from heat inactivation and protease attack by high salt (at least 200 mM) concentrations.

We have proposed that *E. coli* RNAase P is composed of both protein and RNA [6,7]. Indeed, the *E. coli* RNAase P has been separated into RNA and protein, neither of which exhibits enzymatic activity but RNAase P activity can been reconstituted by mixing the separated RNA and protein components [12]. Furthermore Koski [5] has demonstrated inactivation of KB cell RNAase P with micrococcal nuclease, which suggests, but does not prove, an involvement of RNA with the eucaryotic RNAase P as well. As pointed out by Koski [5] the differences between the procaryotic and eucaryotic RNAase P noted above suggest that the putative RNA components are less tightly bound to the eucaryotic enzyme than to *E. coli* RNAase P.

A readily available source of large amounts of eucaryotic RNAase P which

can be used for structural analysis is clearly very important. Whole chick embryos meet these requirements fairly well. Chick embryos have been successfully used for isolation and characterization of two enzymatic activities which degrade double-stranded RNA [13]. Results from chick embryo heart tissue appear even more encouraging in that crude extracts appear almost of degradative nuclease activity, an important advantage in attempts to purify specific endoribonucleases like RNAase P.

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