A NEW METHOD USING 'PROTEINASE K' TO PREVENT mRNA DEGRADATION DURING ISOLATION FROM HeLa CELLS

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SUMMARY:

Diethyl pyrocarbonate (DEP) degrades RNA in a time-dependent reaction at neutral pH with the production of acid-soluble nucleotides. In spite of its inactivating effect on ribonuclease, diethyl pyrocarbonate present during isolation induces considerably degradation of pulse-labeled mRNA of HeLa cells. This can be explained by the above-mentioned reaction.
A new method was developed using 'proteinase K' to inactivate endogenous ribonuclease leading to high specific activities of mRNA extracted from pulse-labeled HeLa cells.

INTRODUCTION:

One of the most critical factors in the isolation and determination of RNA in an undegraded form is the elimination of ribonuclease-catalyzed hydrolysis. Since no satisfactory method for a complete inactivation of ribonuclease was available (the 'hot phenol' method leads to partial degradation of RNA (1), and does not inactivate ribonuclease completely (2,3); betonite is ineffective in completely suppressing ribonuclease action (4)), SOLYMOSY et al. introduced diethyl pyrocarbonate (DEP) as an inactivator of ribonuclease for the isolation of undegraded nucleic acids (5,4). RNA extracted from barley in the presence of DEP had a higher template activity for stimulation of in vitro protein synthesis than RNA extracted by other methods (5). However, when we applied DEP for the isolation of HeLa polysomal RNA, a considerable degradation was observed in comparison to the control values. Recently, a reaction of DEP with nucleic acid components leading to adenine destruction (6) and loss of biological activity (7) has also been observed. We decided therefore to try the use of a new mold proteinase (proteinase K) (8) as a ribonuclease inactivating agent.

This enzyme is available in pure form and exhibits a high affinity for proteins as well as a broad action spectrum. The experiments described below demonstrate the superiority of this method for the isolation of undegraded mRNA.

MATERIAL AND METHODS:

Diethyl pyrocarbonate (DEP 'Baycovin') was a gift of Farbenfabriken BAYER, Leverkusen. Protienase K, chromatogr. pure, isolated from 'tritirachium album limber', was kindly supplied by Dr. H. Lang, Fa.E.MERCK, Darmstadt. Ribonuclease A (Type I-A, bovine pancrease, 5 x cristallized) was obtained from SIGMA Chem. Comp., St. Louis.

HeLa S3 Cells were propagated in suspension culture acc. to (11)

Ribonuclease activity was determined as described earlier (8) with slight modifications. A solution of 0.3% purified (9) yeast-RNA (BOEHRINGER, Mannheim) was incubated in 0.01 M Tris, pH = 7.4, 0.01 M $MgCl_2$, 0.0075 M EDTA, at 37°C. At given times, 0.5 ml of the sample were precipitated with 4.5 ml of ice-cold 'precipitating reagent' (0.63 mM lanthanum nitrate, 11.8 mM Mg^{2+}, 69 mM acetate buffer, pH = 5.5, and 52% (v/v) ethanol), centrifuged and the supernatant was measured for absorbance at 260 nm or for 'acid soluble phosphate' acc. to (9).

Isolation of polysomal RNA from pulse-labeled cells: 1 mC ^{3}H-uridine (uridine-5-T, spec. radioactivity = 30 - C/mmole, The Radiochemical Center, Amersham, England) were added to 1800 ml suspension culture (6 x 10^5 cells/ml) for 60 minutes. Cells were harvested after cooling and addition of cycloheximide (final concentration = 10^{-4}M) by centrifugation (2000 x g , 15 min), and broken up in the presence of cycloheximide acc. to (11). Polysomes were isolated from the postmitochondrial supernatant acc. to KRAMER and HILZ (12).
RNA extraction was performed by a modification of Penman's method (13) at 25°, without heating the phenol to 60°C. Polysomes were incubated for 60 minutes at 25° with DEP or Proteinase K when indicated, and immediately subjected to cold (25°) phenol extraction. The ethanol-precipitated RNA was analyzed by sucrose gradient centrifugation as described by Penman (13).
In the case of a partial degradation of polysomes by added ribonuclease, the isolated polysomes were incubated with ribonuclease (0.01 ug/ml) at 25° for 20 min (cf.12), and cooled immediately. Two aliquots were subjected to DEP or proteinase K action for another 60 min at 25°, while the control remained at 0° to arrest ribonuclease action. Then phenol extraction and sucrose gradient analysis were carried out. Determination of RNA label was performed with the filter paper method of BOLLUM (14).

RESULTS AND DISCUSSION:

1. Degradation of RNA by diethyl pyrocarbonate (DEP)

DEP has been used to inactivate ribonuclease in order to preserve sensitive RNA structures from hydrolysis. However, when ribonuclease inactivation by DEP was analyzed in the presence of RNA, a dose-dependent hydrolysis of RNA was observed in addition to the inactivation of the enzyme (fig.1). DEP values in the presence of ribonuclease were slightly higher than the control without ribonuclease mainly due to an initial reaction of the enzyme before inactivation was completed. The dose-dependent splitting of RNA could also

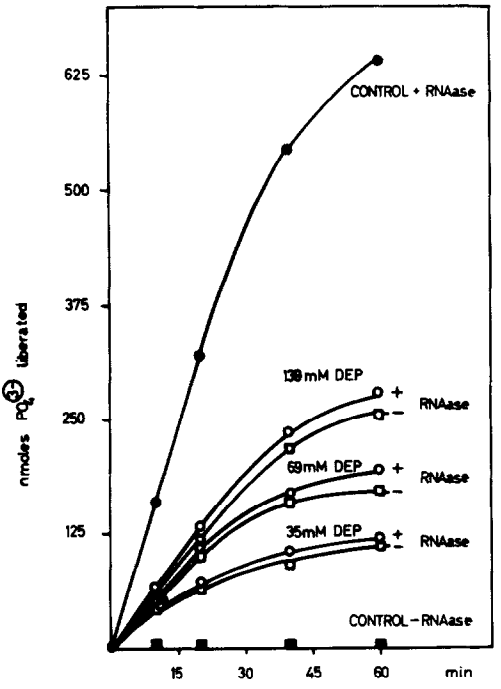

Figure 1:
Hydrolysis of RNA in the presence of DEP (diethyl pyrocarbonate) in spite of ribonuclease inactivation.-

Purified yeast RNA was incubated with different concentration of DEP in the presence or absence of pancreatic ribonuclease (1×10^{-2} ug/ml) under the conditions of the ribonuclease test (see methods). 0.5 ml aliquots were precipitated at the times indicated. Total phosphate was analyzed in the supernatant as described under methods.

be followed by the determination of UV-absorbing non-precipitable material formed during incubation at 37° (not shown). DEP also lead to an extensive degradation of mRNA extracted from HeLa cell polysomes (see below and fig.3). These secondary effects of DEP render to compound inpracticable as a tool for the isolation of undegraded RNA.

2. The Use of Proteinase K for the Isolation of undegraded RNA

A new method was developed which allows rapid and complete inactivation of endogenous and exogenous ribonuclease. It is based on the degradation of even minute amounts of ribonuclease by a mold protease (proteinase K) available in electrophoretically pure form. Fig.2 demonstrates the rapid inactivation of ribonuclease in the presence of substrate. Inactivation was instantaneous with 200 µg of proteinase K.

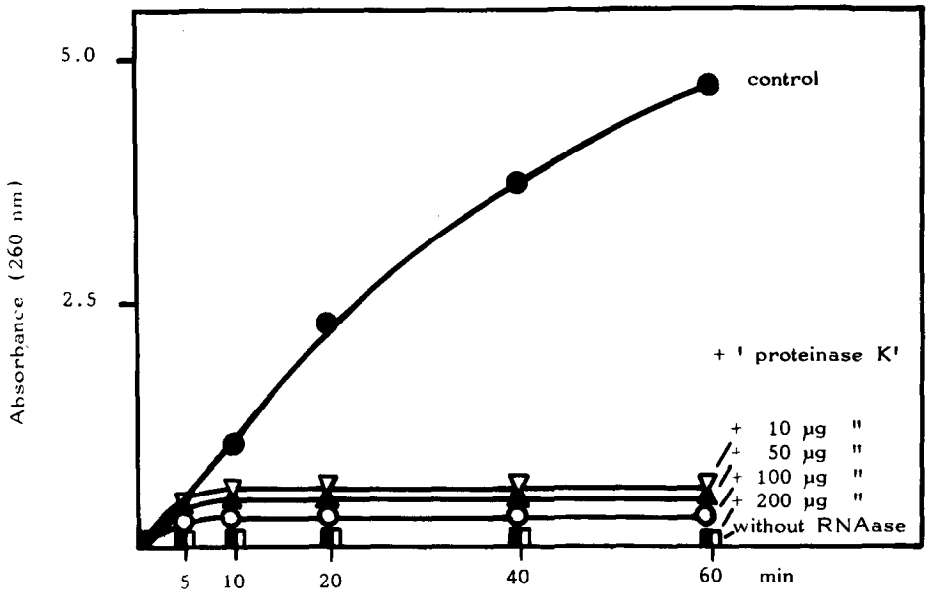

Figure 2:
Inactivation of ribonuclease activity by different concentrations of proteinase K .-

Purified yeast RNA was incubated at 37° in the presence of pancreatic ribonuclease (1×10^{-2} µg/ml) under the conditions of the ribonuclease test. Different concentrations of proteinase K were added at zero time (0°) where indicated. Aliquots were taken at the indicated times, and remaining RNA was precipitated as described. UV-absorbing split products were determined at 260 mµ in the supernatant.

The degrading effect of DEP as well as the clear superiority of proteinase K in the isolation of polysomal RNA is documented in fig.3. Pulse-labeled (60 min ^{3}H-uridine) HeLa cells were broken

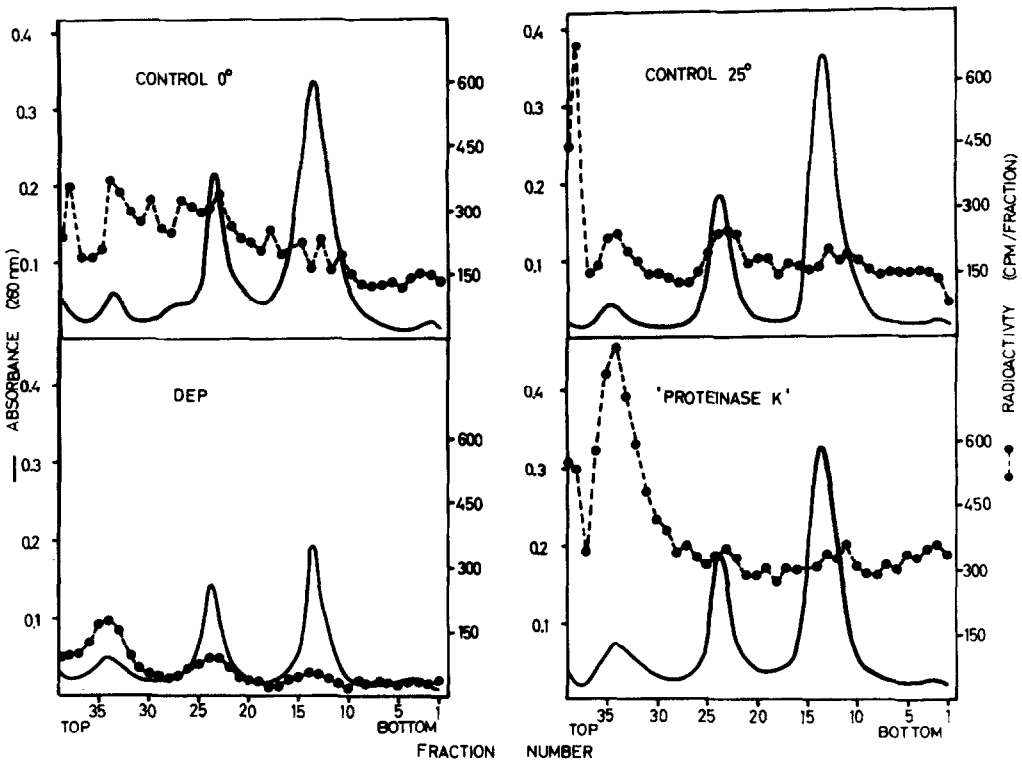

Figure 3:

Sucrose gradient analysis of polysomal RNA isolated from HeLa S3 cells after pulse-labeling with ^{3}H-uridine.-

HeLa S3 cells in suspension culture (1800 ml, 6 x 10^5 cells/ml) were labeled with 1 mC ^{3}H-uridine for 60 min. Polysomes were isolated as described in methods. Aliquots of 10 A_{260}-units/2 ml (6000 cpm/A_{260}-unit) were preincubated for 60 min with the following additions.
A. H_2O (control, 0°). B. H_2O (control, 25°). C. DEP (33 mM, 25°). D. proteinase K (200 ug/ml, 25°). After 'cold phenol' extraction, RNA was precipitated with ethanol, and precipitates were dissolved in 1.0 ml of sterile water. 0.5 ml were layered on a linear gradient (5 to 20% sucrose in 0.01 M Tris, pH = 7.4, 0.1 M NaCl, 0.001 M EDTA), and centrifuged for15 hrs at 25 000 rpm and 4° in a Spinco SW 40 rotor. A_{260} was monitored automatically, and fractions of 0.3 ml were collected. TCA-insoluble material was analyzed for radioactivity by the filter paper method.

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and free polysomes isolated from postmitochondrial supernatant as described under methods. To the pellet were added DEP, proteinase K, or water as indicated. One control (H_2O) was run at 25°,

the other at 0°. After 60 min, polysomes were subjected to cold (25°) phenol extraction. RNA was analyzed by sucrose gradient analysis. While incubation of polysomes at 25° produced a partial degradation of mRNA due to endogenous ribonuclease (B versus A), DEP led to a considerable loss of (labeled) mRNA as well as of rRNA (compared to the controls). In clear contrast, proteinase K preserved the apparently highly sensitive mRNA fractions. Especially remarkable is the presence of high molecular weight mRNA ($\geq$ 28 S), and the high radioactivity peak in the region of 5 S RNA.

The complete stop of ribonuclease activity by proteinase K was also seen in polysomes preincubated with very low ($<$ 0.01 µg/ml) ribonuclease concentrations (not shown). While mRNA had been degraded nearly to completion by this pretreatment of the polysomes, degradation of 18 S and 28 S RNA during the isolation of RNA was indicated by the asymmetric shape of the peaks, except in the case where proteinase K was added after the preincubation to inactivate ribonuclease. There was also a much better preservation of the radioactivity peak in the region of 4 - 5 S.

The results demonstrated the possibility to isolate undegraded RNA from polysomes. When combined with the addition of cycloheximide to prevent 'running' out' of mRNA during isolation of cells and preparation of polysomes (cf. 11, 12), a reasonable approach to a true in vivo situation can be achieved.

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ERRATUM

Volume 43, Number 2 (1971) in the Communication, "The Identification of the Special Glutamic Acid Residue Essential for Activity of Cobratoxin", by C. C. Chang, C. C. Yang, Masayuki Kurobe, Kenji Nakai, and Kyozo Hayashi, pages 429-434, the last 3 authors' names, Masayuki Kurobe, Kenji Nakai, and Kyozo Hayashi, were left out of the Table of Contents and the Author Index.