ENZYMATIC BREAKDOWN OF RAPIDLY LABELED NUCLEAR RNA AND ITS INHIBITION BY CYTOPLASMIC SOLUBLE FRACTION

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Polynucleotide phosphorylase has been implicated in the breakdown of rapidly labelled RNA in mammalian cell nuclei (4) and of messenger RNA in bacteria (11). In these studies, the reaction products examined have been acid soluble. The present communication describes the breakdown of rapidly labeled, high molecular weight nuclear RNA to products in the 4-6 S size range.

Balb/C mice bearing RPC 20 plasma cell tumors (6) were killed 30 minutes after the intraperitoneal administration of 90 μc H^3-uridine. The tumors were rapidly removed, chilled, and homogenized in 3 volumes of a 0.25 M sucrose solution containing 4 mM $MgCl_2$. Homogenates were filtered through a bed of 3 mm beads to remove large tissue clumps. The nuclei were sedimented by centrifugation of the filtrates at 200 x g for 15 minutes, purified by 2 washings in the homogenizing medium followed by two additional washings in 0.25 M sucrose and 2 mM $MgCl_2$, and finally resuspended in the latter medium. In the incubation

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experiments, nuclei from 0.5 g tissue were shaken at 37° C in 4 ml of a medium consisting of 150mM NaCl, 75 mM Tris Cl (pH 7.4), 2 mM $MgCl_2$ and 0.125 M sucrose. In some cases 2 mM $CaCl_2$, instead of 2 mM $MgCl_2$, was used for incubation (see Fig. 2).

The incubation mixtures were cooled to 0° C, diluted to 10 ml, the RNA was extracted at pH 5.3 by the phenol-SDS* method of Scherrer and Darnell (9) with the following modifications which were found to give optimal results with plasma cell nuclei: 1) the concentration of SDS was increased from 0.5 to 1.0% and 2) the initial phenol extraction at 60° C was prolonged from 3 to 7 minutes. Polyvinylsulfate was not used in the extraction procedure.

RNA was precipitated with 2 volumes of cold absolute ethanol (9) and redissolved in 10 mM Tris Cl (pH 7.4) containing 1 mM $MgCl_2$. To effect complete equilibration with this buffer and remove possible nuclease contamination, the solutions were passed through a mixed bed column composed of Sephadex G-50 and carboxymethyl cellulose (45-100 mesh, 0.5 meq. per gram) in a weight ratio of 10:1.**

Two ml of the RNA solutions (column eluates) usually containing 5-8 A_{260} units were layered on 5-25% linear sucrose gradients (volume 27 ml, containing 10 mM Tris Cl, pH 7.4, and 1 mM $MgCl_2$) and centrifuged for 10 hours at 25,000 RPM in the SW-25 head of the Model L Spinco ultracentrifuge. Fractions were collected, read in a Beckman DU spectrophotometer and counted in a liquid scintillation system employing Bray's solution (1).

The specific activities of the phenol-extracted RNA were similar to those of the total nuclear RNA extracted by the Schmidt-Thannhauser procedure and recoveries ranged from 50-70%. DNA represented less than 3% of the nucleic acid and 0.1% of the radioactivity in the phenol extracted preparations.

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* Abbreviations: SDS, Sodium Dodecyl sulfate; Tris Cl, Tris (hydroxymethyl) aminomethane buffer; PCMB; p-chloromercuribenzoate.

** Private communication from E. A. Peterson and E. L. Kuff

RESULTS

Figs. 1A, 2A and 2D show the sedimentation patterns of the RNA extracted from non-incubated nuclei. The three major optical density peaks were identified as S-RNA and two species of ribosomal RNA by reference to other gradient analyses (cf Figs. 1C and 4A). With the 30 minute incorporation period used in these experiments, a major portion of the label was associated with RNA that sedimented more rapidly than ribosomal RNA.* Similar distributions of radio-activity have been observed in studies of pulse-labeled RNA from other mammalian systems (5,10,12). Significant amounts of absorbancy were also present in the lower portions of the gradient.

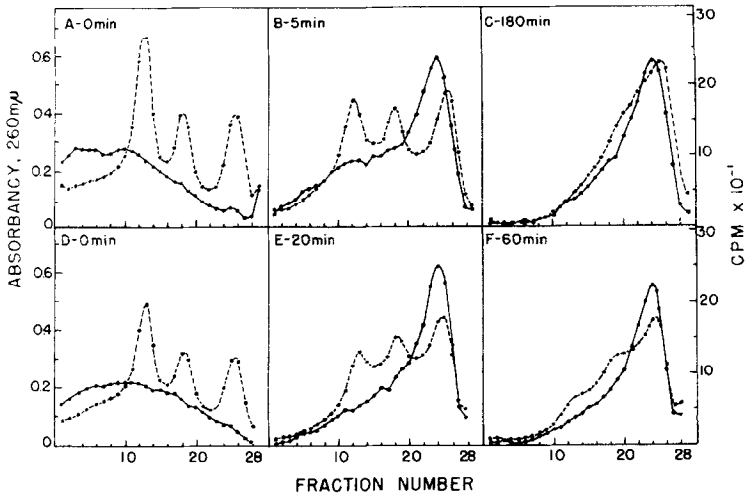

Fig. 1 Sucrose density gradient profiles of plasma cell tumor RNA. Homogenate and nuclei were prepared in the presence of Mg ion from tumors labeled in vivo for 30' with H^3-uridine. RNA was extracted from A) nuclei stored at 0°C, B) nuclei incubated at 37°C for 20' C) whole homogenate incubated at 37°C for 20'. Incubations were done in Mg-NaCl medium (see text).

The two main optical density peaks in Fig. 1C (Fractions no. 14 and no. 19) are derived mainly from cytoplasmic ribosomal RNA.

In all gradients, fraction no. 1 represents material collected from the bottom of the gradient, no. 28 material from the top. ----A260; _____c.p.m.

* A significant proportion (up to 25%) of radioactivity from unincubated controls was recovered from the bottom of the gradient tubes after sampling had been completed. This pelletized RNA was of high specific activity and was not present in RNA extracted from incubated nuclei.

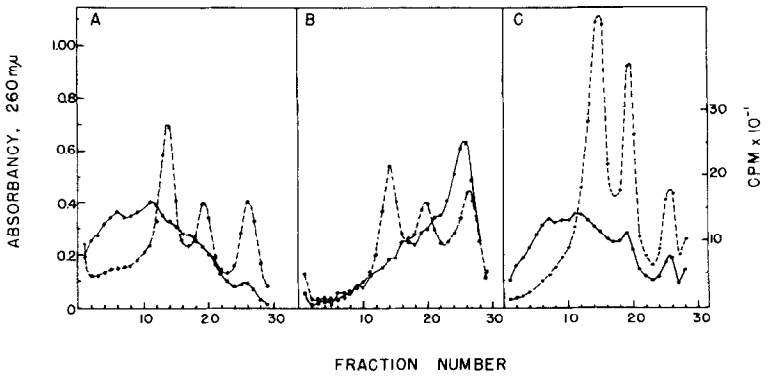

Fig. 2 Sucrose density gradient profiles of RNA extracted from nuclei isolated in 0.25 M sucrose + 3 mM $CaCl_2$ and incubated in a $CaCl_2$ - NaCl medium (see text). A) unincubated (0°C) control for B and C, B) 5' incubation at 37°C, C) 180' incubation at 37°C, D) unincubated (0°C) control for E and F, E) 20' incubation at 37°C. ----A260; _____c.p.m. F) 60' incubation at 37°C.

Incubation of the nuclei in a medium containing Mg^{++} (Fig. 1B) or Ca^{++} (Fig. 2) resulted in a rapid reduction of the sedimentation rate of the labeled RNA. The peak of radioactivity now appeared in the 4-6S region of the gradients (as judged by reference to the S-RNA peak). After 5 minutes incubation, a bimodal distribution of label was evident, but this was lost by 20 minutes. The kinetics of the change is illustrated in Fig. 3 where the proportion of the counts in the upper regions of the gradients (fractions 20-28) is plotted against incubation time. The shift of radioactivity was essentially complete in 1 hour. During the period of most rapid change (i.e., up to 20 minutes incubation), there were only small losses in specific activity (10%) and total RNA (15%). This shift in radioactivity was accompanied by a rapid loss of optical density in the lower portion of the gradient. Breakdown of ribosomal RNA also occurred (Fig. 2), but at a slower rate. The degradation of ribosomal RNA to 4 S-RNA under a variety of conditions has been observed by others (2,7,13).

The above reactions were not dependent upon the high ionic strength in the incubation mixtures, since similar reductions in size of rapidly

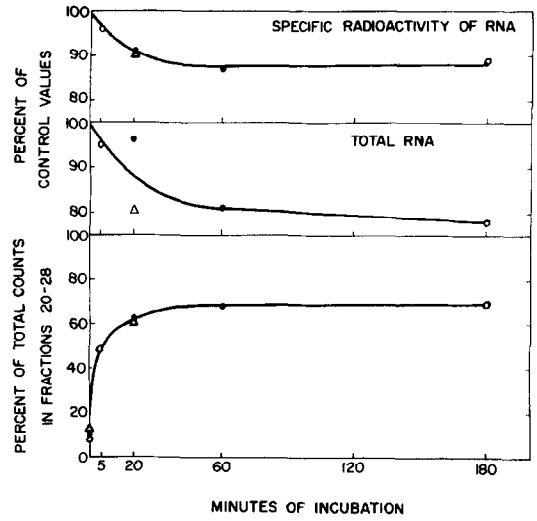

Fig. 3 Quantitative changes in nuclear RNA during in vitro incubation at 37°C. Points are from values obtained for phenol-extracted RNA from the following figures: O-Figs. 2A, 2B and 2C; -Figs. 2D, 2E and 2F; Δ - Figs. 1A and 1B.

labeled RNA were found when nuclei were incubated in 2 mM $MgCl_2$ and 10 mM Tris Cl (pH 7.4).

In an effort to localize the reaction products, nuclei were incubated in both high and low ionic strength media, and then sedimented and assayed for acid precipitable radioactivity. In the high salt medium, 77% and 63% of the counts (compared with non-incubated controls) were present in the nuclei after 5 and 20 minutes incubation, respectively. On the other hand, 96% and 91% of the counts were recovered in the nuclei incubated in the low salt medium for the same times. These data suggest that the breakdown of high molecular weight RNA occurs within the nucleus, and that the end products are easily extractable in the high salt medium.

The above results were consistent with the action of an endonuclease which preferentially attacked high molecular weight RNA in the tumor nuclei. Powerful inhibitors of alkaline ribonuclease are known to occur in the soluble fraction of mammalian cells (8). Fig. 1C shows that when

whole homogenate was incubated for 20 minutes, breakdown of rapidly labeled RNA did not occur. A similar inhibition was observed when radioactive nuclei were incubated in the presence of non-labeled cytoplasmic soluble fraction (Fig. 4A). Breakdown of the high molecular wieght RNA occurred rapidly, however, in the presence of the cytoplasmic soluble fraction which had been pretreated with 4×10^{-4} M PCMB (Fig. 4B), a sulfhydryl reactant known to block ribonuclease inhibitors in a number of other tissues (8).

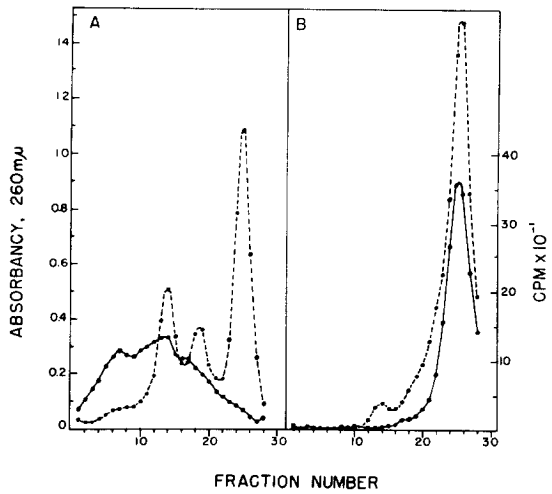

Fig. 4 Sucrose density gradient profiles of RNA extracted from nuclei incubated at 37°C for 20' in Mg-NaCl medium with A) cytoplasmic soluble fraction (2 1/2 hr., 105,000 xg supernatant). B) cytoplasmic soluble fraction pretreated with PCMB (final concentration 4×10^{-4}M). Soluble fraction from 0.5 g tissue used in each incubation mixture. The main peak in Fig. 4A represents chiefly the cytoplasmic soluble RNA. ----A260; _____c.p.m.

Polynucleotide phosphorylase does not appear to be responsible for the changes observed in the present experiments, where marked reductions in sedimentation rate were attended by minimal losses in specific and total radioactivity. There is no necessary inconsistency between our results and those of Harris (4) using HeLa cell nuclei, since 1) our incubations were carried out under conditions (no added P_i)

that would tend to minimize phosphorylase activity and 2) the experiments of Harris did not eliminate a reduction in size of the RNA prior to the breakdown by phosphorylase.

In summary, the results presented in this communication indicate that there is an enzyme present in the nuclei of plasma cell tumors which, when uninhibited, degrades rapidly labeled, high molecular weight nuclear RNA to products of 4-6 S size. The action of the enzyme is strongly inhibited in the presence of cytoplasmic fraction and this inhibition can be reversed by the addition of PCMB.

The rapidly labeled RNA of mammalian nuclei is thought to represent both messenger RNA and precursors of ribosomal RNA (3,10). The formation of 28 and 16 S ribosomal RNA'S presumably involves cleavage of the larger molecules at selected points. Studies are in progress to determine whether this conversion can be effected in isolated nuclei under proper conditions of inhibition by soluble fraction.

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