EFFECT OF PUROMYCIN ON RNA SYNTHESIS IN HELA CELLS*

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Sedimentation analyses of RNA from HeLa cells have revealed that rapidly-labeled RNA sediments faster in a sucrose gradient than ribosomal RNA (Scherrer and Darnell, 1962). This RNA fraction is confined to the nucleus and its synthesis is prevented by a low level (0.01 μg/ml) of actinomycin D (Tamaoki and Mueller, 1962). Recent papers by Perry (1963) and Scherrer et al. (1963) indicate that at least two kinds of RNA are present in the rapidly sedimenting fraction: (1) a precursor RNA which converts into ribosomal RNA in the presence of actinomycin D, and (2) DNA-like RNA which is capable of forming a hybrid with HeLa cell DNA and stimulates amino acid incorporation in a cell-free E. coli system.

In this paper, the effect of puromycin on RNA metabolism in HeLa cells will be presented. The data illustrate that the synthesis and turnover of a fraction of the heavy RNA continued in the presence of puromycin, whereas the introduction of guanine-8-C^{14} into other classes of RNA (28 and 16s) was blocked by puromycin.

Experimental

Spinner cultures were used in all experiments except those in which it was desirable to change the medium rapidly without sedimentation and resuspension of the cells; in such cases monolayer cultures in prescription bottles were employed. The labeling of the cells was accomplished by addition of guanine-8-C^{14} (8×10^{-6}M, 6 μc/μmole) or uridine-H^3 (0.033 μc/ml,

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6.8 μc/μmole). Puromycin was used at a concentration of 20 μg/ml. Cells were harvested by centrifugation. In the case of monolayer cultures, the cells were first trypsinized.

The nucleic acids were prepared by the phenol extraction method as described previously (Tamaoki and Mueller, 1962). A concentration of 0.1% dextran sulfate was maintained throughout the entire preparative procedure; this appeared to protect RNA from enzymatic degradation and resulted in reproducible preparations.

Centrifugation was carried out in the SW39 rotor at 35,000 rpm for 3 hrs. at $4^{\circ}C$ through a sucrose gradient of 5 to 30% in the presence of 0.001 M $MgCl_2$ and 0.01 M tris buffer, pH 7.4. Under these conditions the rapidly labeling RNA sediments as a broad single peak (approx. 35-45s) ahead of the ribosomal RNA area (28s). These conditions were employed to achieve best resolution of the heavy RNA area; the 28s and 16s RNA are not well resolved. After centrifugation, fractions of 15 drops each were collected and analyzed for RNA as described previously (Tamaoki and Mueller, 1962).

The radioactivity incorporated into the total RNA of the cell was measured as the RNAse released count from the perchloric acid (PCA, 2.5%) insoluble residue. For this purpose the residue from 5×10^6 cells was suspended in 1 ml of 0.06M $KHCO_3$, digested for 30 minutes at $37^{\circ}C$ with 20 μg pancreatic RNAse and re-precipitated with PCA. The same procedure was used for the assay of RNA in the phenol-extracted nucleic acid preparations except 1 mg of bovine serum protein was added as a co-precipitant.

Results

Fig. 1 shows the effect of puromycin (20 μg/ml) on the incorporation of guanine-8-C^{14} into HeLa cell RNA as determined on the phenol-extracted RNA and on PCA-precipitated cell residues. The counts in RNA prepared by the phenol-extraction method were about 80% of the values obtained by direct PCA precipitation. In both cases, puromycin suppressed the incorpora-

tion to one-half of the control level in the first 30 minutes. Thereafter the counts increased only slightly in the presence of puromycin.

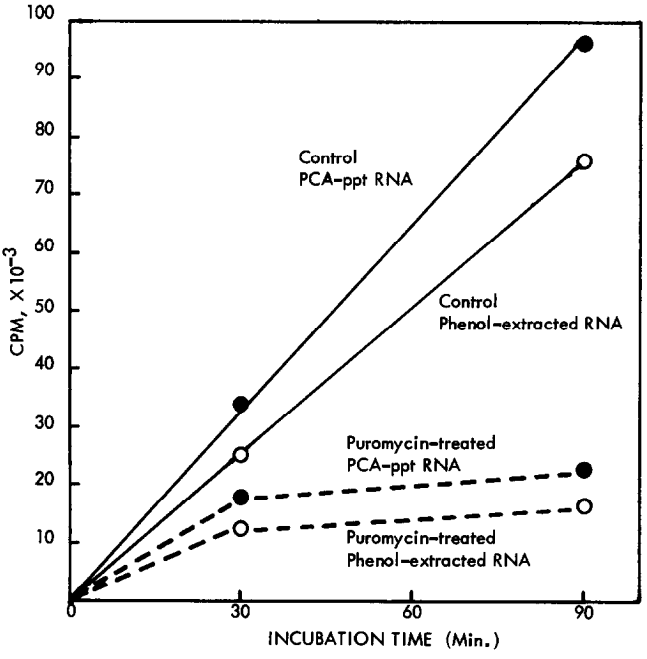

Figure 1. Effect of puromycin on incorporation of guanine-8-C^{14} into HeLa cell RNA.

●——● : Control cells, PCA-precipitated RNA
○——○ : Control cells, Phenol-extracted RNA
●- - -● : Puromycin-treated cells, PCA-precipitated RNA
○- - -○ : Puromycin-treated cells, Phenol-extracted RNA

Puromycin at the concentration used in these experiments has been shown previously to inhibit protein synthesis in HeLa cells by 90 per cent (Mueller et al., 1962).

Sedimentation analyses showed that the radioactivity in the puromycin-treated cells was confined primarily to the heavy RNA fraction throughout the entire experimental period (Fig. 2B). In contrast the control cultures (Fig. 2A) exhibited a progressive labeling of the ribosomal-type RNA. The existence of a ribosomal RNA precursor and its conversion to ribosomal RNA through an actinomycin D-insensitive step, similar to that described by Perry (1963) and Scherrer et al. (1963), was also demonstrated in the control cultures (Fig. 3).

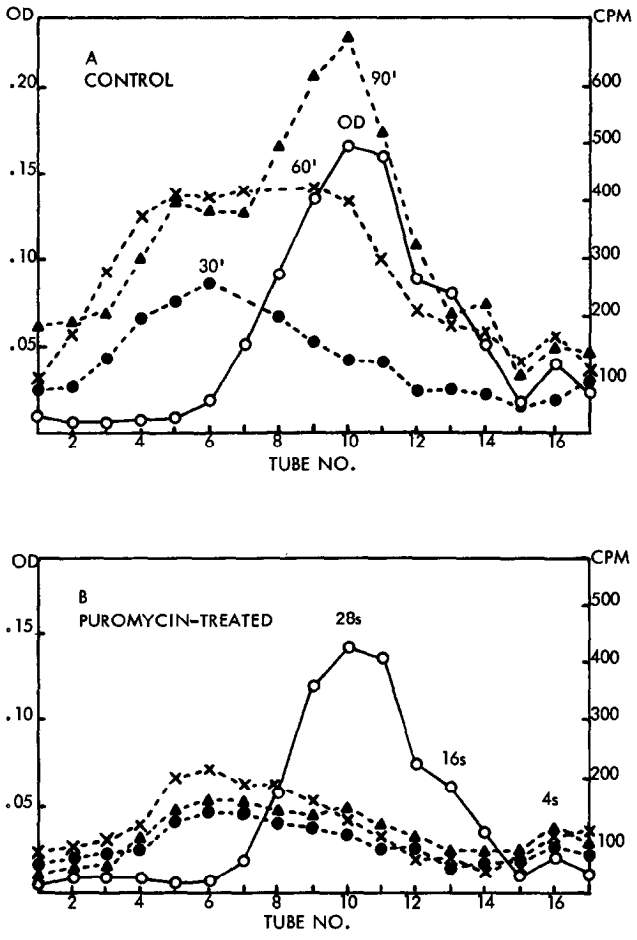

Figure 2. Sedimentation pattern of newly synthesized RNA from control and puromycin-treated cells.

HeLa cells were labeled with guanine-8-C^{14} for 30,60 and 90 minutes in the presence of puromycin (20 µg/ml). RNA was extracted by the phenol method and sedimented in a 5 - 30% sucrose gradient for 3 hrs. Optical density patterns of RNA from all cultures were very similar.

●---● : CPM, 30 min. labeling
×---× : CPM, 60 min. labeling
▲---▲ : CPM, 90 min. labeling
O——O : OD at 260 mµ, 30 min. labeling

In all experiments the labeling of the heavy RNA fraction in the presence of puromycin attained a maximum in less than 30 minutes and remained relatively unchanged in amount during the ensuing period. Even pretreatment

of the cells with puromycin for 30 minutes prior to the addition of radioactive guanine yielded the same maximum labeling pattern. These results suggested that only a certain quantity of the heavy RNA was synthesized in the presence of puromycin and that this fraction was subject to rapid turnover.

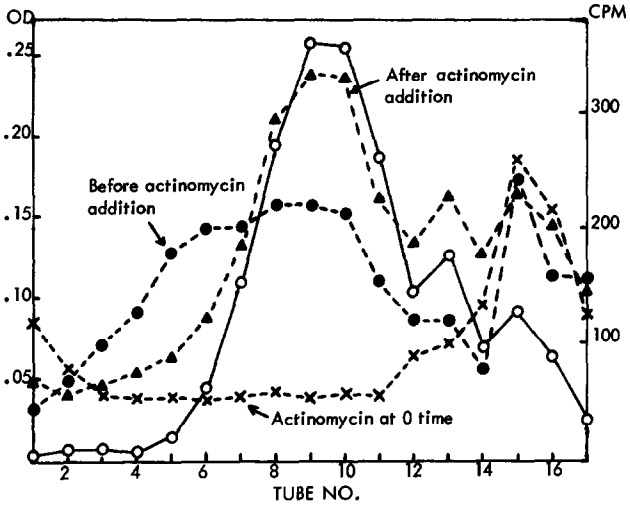

Figure 3. Change of labeling pattern of newly synthesized RNA following the addition of actinomycin D.

A HeLa cell culture was labeled with uridine-H^3 for 1.5 hrs., and a portion of the culture was withdrawn for extraction and sedimentation of the RNA. Actinomycin D (0.1 μg/ml) was added to the rest of the culture and incubation was continued for additional 1.5 hrs. prior to RNA analysis. A control culture received both actinomycin D and uridine-H^3 at zero time and was incubated for 3 hrs.

●----● : CPM, cells were labeled for 1.5 hrs.
▲----▲ : CPM, cells were labeled for 1.5 hrs., and then incubated for 1.5 hrs. in the presence of actinomycin D.
×----× : CPM, cells were labeled for 3 hrs. in the presence of actinomycin D.
O——O : OD at 260 mμ of RNA from cells labeled for 1.5 hrs. OD patterns of RNA from all cultures were very similar.

In further studies cells were pre-treated with puromycin for 15 minutes and then incubated with guanine-8-C^{14} for 45 minutes in the presence of puromycin (total puromycin treatment was 60 minutes) to assure the labeling of the heavy RNA in the absence of protein synthesis. Replacement of the

puromycin medium with fresh medium resulted in the rapid accumulation of radioactivity in the ribosomal RNA area (Fig. 4) demonstrating the reversibility of the puromycin effect. The presence of actinomycin D in the new medium, however, prevented majorly this appearance of isotope in the ribosomal RNA fractions (Fig. 4). Accordingly it would appear that labeling of the ribosomal type RNA on reversal of the puromycin effect by the medium change involves the de novo synthesis of this RNA from a DNA template, a process which has been shown to be blocked by actinomycin D (Reich et al., 1961 and 1962; Goldberg and Rabinowitz, 1962; Hurwitz et al., 1962).

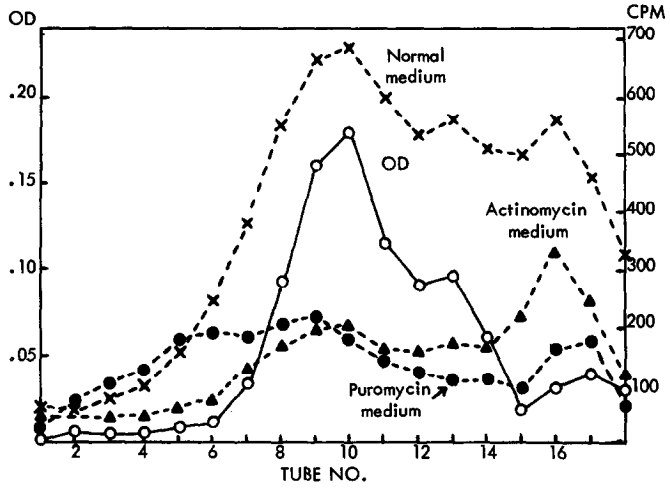

Figure 4. Reversal of puromycin effect by medium change and effect of actinomycin D.

The cultures were pre-treated with puromycin (20 µg/ml) for 15 min. and then labeled with guanine-8-C^{14} for 45 min. in the presence of puromycin. After removal of the labeling medium the cultures were further incubated for 1.5 hrs. in the new media shown below:

- ●----● : **CPM, medium for the second incubation contained puromycin (20 µg/ml).**
- ▲----▲ : **CPM, medium for the second incubation contained actinomycin D (0.1 µg/ml).**
- X----X : **CPM, medium for the second incubation was normal medium.**
- O——O : **OD at 260 mµ of RNA from cells incubated in the puromycin-medium (second incubation). OD patterns of RNA from all cultures were very similar.**

It was also observed that in the presence of actinomycin D the amount of isotope in the heavy RNA fraction decreased strikingly with a concomitant

increase of the labeling of the 4S area in the sedimentation pattern (Fig. 4). Whether the accumulation of counts in the 4S area involves the transfer RNA or a degradation product of the heavy RNA remains to be determined.

Based on the present data we propose the concept shown in Fig. 5 with reference to the synthesis of ribosomal type RNA. It is suggested that nucleotides are first incorporated into a polymeric precursor A which matures by a puromycin sensitive process to precursor B. In the normal HeLa cultures a significant amount of precursor B accumulated which in turn can be converted by a yet unknown but actinomycin D-insensitive process to the ribosomal type RNA (28 and 16s).

Fig. 5 Synthetic pathway of ribosomal RNA and blocking sites of puromycin and actinomycin D.

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