A REGULATORY ROLE OF PROTEIN SYNTHESIS ON THE ACTIVITY OF RNA POLYMERASE OF HELA NUCLEI[1]

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Puromycin and cycloheximide are powerful inhibitors of protein synthesis in mammalian cells (Mueller, et al., 1962; Zimmerman, 1963; Young, et al., 1963; Ennis and Lubin, 1964). This report describes the acute depression of RNA synthesis that accompanies the inhibition of protein synthesis by these agents. In partial explanation of this phenomenon, it has been found that pretreatment of exponentially growing cells with amounts of puromycin and cycloheximide which inhibited in vivo synthesis of protein and RNA greatly reduced the RNA polymerase activity of isolated nuclei. The antibiotics had no effect when added in vitro to the nuclear RNA polymerase system. The data suggest that nuclear RNA polymerase activity depends strongly on the synthesis of new protein. This dependency may be important in the regulation of RNA synthesis in mammalian cells.

Methods. HeLa cells growing as suspension cultures in a modified Eagle's medium (Mueller, et al., 1962) were used during exponential growth (3 to 4 x 10^5 cells/ml). For some experiments cells were taken from cultures which had grown to a stationary phase (7 x 10^5 cells/ml). Puromycin (20 μg/ml) or cycloheximide (10 or 20 μg/ml) was added to the cultures 0 to 3 hr before harvest.

To determine the rate of protein synthesis, aliquots of cells were

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incubated for 30 min with 0.4 μC/ml of H^3-L-leucine (5 C/mmole). The activity in extracts insoluble in 2.5% PCA (perchloric acid) was determined by liquid scintillation counting. RNA synthesis in vivo was measured by incubating aliquots of cells for 30 min with 0.25 μC/ml of H^3-cytidine (50 mC/mmole) and determining the amount of radioactivity which RNAase released from the acid-insoluble product. In one experiment (Fig. 1) RNA synthesis was determined with a 10-min pulse with C^{14}-guanine. With this precursor RNAase released more than 85% of the incorporated radioactivity.

To assay the RNA polymerase activity of isolated nuclei, cells were washed once in saline, once in distilled water, resuspended in distilled water at a concentration of 20×10^6 cells/ml, and after 5 min were ruptured by 15 strokes in a Dounce homogenizer. After centrifuging at 900 x g for 10 min, the sedimented nuclei were resuspended in 0.1 M Tris buffer, pH 8.0, with 0.075 M KCl and 0.005 M $MgCl_2$. Aliquots of the nuclear suspension containing 2×10^6 nuclei were transferred to tubes containing the rest of the assay system and were incubated at 37°C. The final concentrations of the constituents were 10^{-1} M Tris (pH 8.0), 7.5×10^{-2} M KCl, 5×10^{-3} M $MgCl_2$, 3×10^{-2} M NaF, 10^{-2} M cysteine, 10^{-3} M adenosine triphosphate, 4×10^{-4} M guanosine triphosphate, 4×10^{-4} M uridine triphosphate, and 10^{-6} M H^3-cytidine triphosphate (H^3-CTP, 1 C/mmole) in a total volume of 1 ml. In some cases 1 μg actinomycin D, 20 to 200 μg puromycin, or 20 to 100 μg cycloheximide were included in the reaction mixture. After intervals of 0 to 30 min the samples were chilled, 1 mg of serum protein was added as carrier, and the RNA was coprecipitated with 4 ml of 2.5% PCA. The precipitate was washed twice with PCA, dissolved in formic acid, and its radioactivity determined.

Results and Discussion. Puromycin or cycloheximide rapidly inhibited incorporation of leucine into total cell protein and guanine into total RNA of HeLa cells (Fig. 1). Cycloheximide blocked 90% of the leucine incorporation almost immediately, and puromycin produced its maximal inhibi-

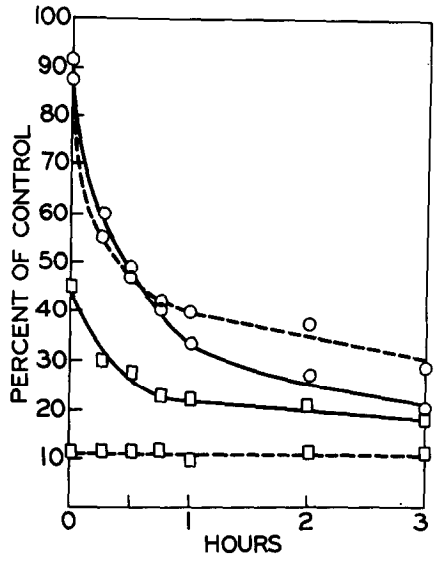

Fig. 1. The effects of cycloheximide (20 μg/ml,---) and puromycin (20 μg/ml,_____) on protein, □ , and RNA, ○ , synthesis in vivo. Radioactivity measured in the acid-insoluble residue (no RNAase treatment) after a 10-min pulse with C^{14}-guanine or H^3-leucine. 5.8 x 10^5 cells/ml.

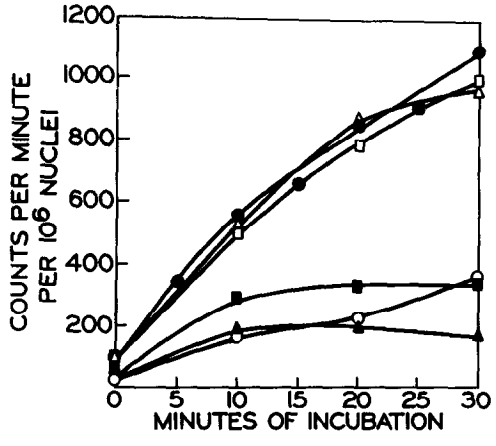

Fig. 2. The time course of the in vitro assay for nuclear RNA polymerase activity. Incubation conditions and media are described in the text. Untreated, ● ; 20 μg/ml puromycin, □ ; 20 μg/ml cycloheximide, △ ; 1 μg/ml actinomycin D ○ ; 20 μg/ml DNAase, ■ ; 20 μg/ml RNAase, ▲ .

tion, 80%, near the end of the first hour of treatment. Within 20 min both agents had halved guanine incorporation into RNA, and RNA synthesis measured by this means continued to fall. Thus two agents which acutely

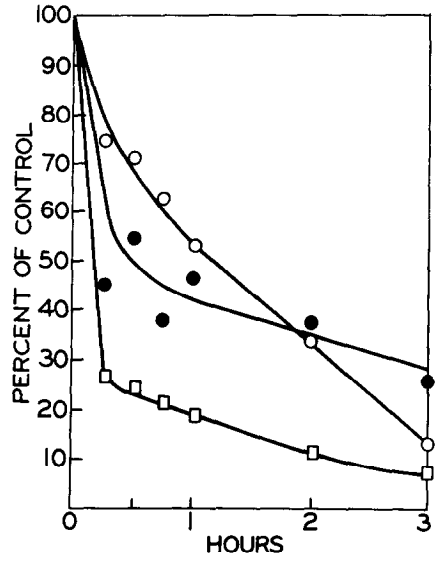

Fig. 3. The effect of 20 μg/ml puromycin on protein and RNA synthesis and the activity of nuclear RNA polymerase. Protein synthesis, □ , measured *in vivo* over 30 min with H^3-leucine; RNA synthesis, ○ , measured by the RNAase-releasable radioactivity following 30 min with H^3-cytidine. RNA polymerase, ● , assayed by the *in vitro* incorporation during 30 min of H^3-CTP into an acid-insoluble product. 3.9 x 10^5 cells/ml.

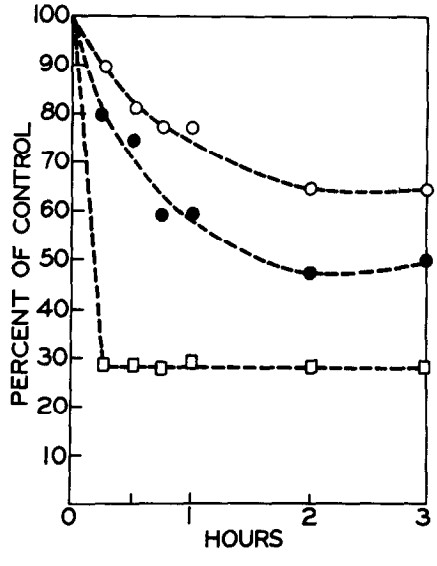

Fig. 4. The effect of 10 μg/ml cycloheximide on protein and RNA synthesis and the activity of nuclear RNA polymerase. Conditions and symbols were as in Fig. 3. 3.2 x 10^5 cells/ml.

inhibit protein synthesis by different mechanisms as shown in cell-free systems (Yarmolinsky and de la Haba, 1959; Allen and Zamecnik, 1962; Siegel and Sisler, 1963; Wettstein, et al., 1964) progressively inhibited RNA synthesis in vivo.

The incorporation of H^3-CTP by HeLa nuclei into an acid-insoluble product was used to assay nuclear RNA polymerase activity. The reaction required all four riboside triphosphates, yielded a RNAase-sensitive product, and was inhibited by 1 μg/ml of actinomycin D. The amount of incorporation was proportional to the number of nuclei. Exogenous DNA did not augment the incorporation, but DNAase greatly depressed the formation of the RNA-like product. Fig. 2 shows the time course of the in vitro reaction, and its sensitivity to RNAase, DNAase, and actinomycin D. Neither puromycin nor cycloheximide, even at 100 μg/ml, inhibited the reaction when added to the incubation mixture.

Although cycloheximide and puromycin did not inhibit RNA polymerase directly, pretreatment of the cells with either compound greatly reduced the level of the enzyme activity in nuclei (Fig. 3 and 4). During the first hour of treatment the polymerase activity fell rapidly; with longer treatment it tended to plateau at 35 to 55% of the level of exponentially growing cells. The initial drop in polymerase activity correlated with the decline in RNA synthesis in vivo.

The growth state of the culture determined the sensitivity of RNA polymerase to puromycin. The RNA polymerase activity of nuclei from cells which had grown to a stationary phase was lower and less sensitive to the in vivo action of puromycin than that of rapidly growing cells (Fig. 5). The level of activity of untreated cells in stationary growth was similar to that of puromycin-treated, exponentially growing cultures, suggesting that the stationary cultures had already lost that portion of polymerase activity which depended on the continued synthesis of protein.

The present studies also showed that the intact state of the nucleus

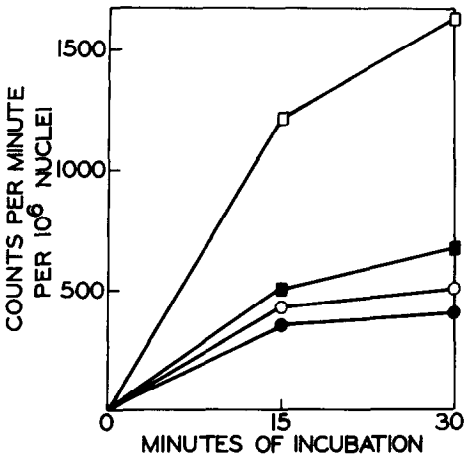

Fig. 5. The effect of stationary growth conditions on sensitivity of RNA polymerase activity to 20 μg/ml puromycin. Nuclei isolated from exponentially growing, untreated cultures, 3.3 x 10^5 cells/ml, □ ; from exponentially growing cultures treated with puromycin for 60 min, 3.3 x 10^5 cells/ml, ■ ; from untreated cultures in a stationary growth phase, 6.9 x 10^5 cells/ml, ○ ; from stationary cultures treated for 60 min with puromycin, 6.9 x 10^5 cells/ml, ● .

was essential for optimal polymerase activity. Mild sonication of nuclei suspended in the incubation buffer destroyed 90% of the RNA polymerase activity. The disruptive treatment may have increased interaction between polymerase and an *in situ* inhibitor, or may have dispersed an essential activating protein. The residual activity, however, no longer reflected the pretreatment of the cells with puromycin. This finding may explain Holland's (1963) observation that pretreatment of HeLa cells with puromycin had little effect on the activity of DNA-primed, aggregate enzyme from lysed nuclei.

Taken together these findings suggest that puromycin and cycloheximide influence RNA synthesis *in vivo*, not by inhibiting directly the enzymatic polymerization of the triphosphates, but by inhibiting the synthesis of some protein which is utilized in another aspect of RNA synthesis as it occurs in the nucleus. While not excluded by these experiments, it appears unlikely that the reduction of polymerase activity results primarily from the blocked synthesis of the enzyme. Its turnover rate would have to be unusually fast to account for the rapid decline of activity observed during the first hour of restricted protein synthesis. Furthermore, it would be difficult to explain on this basis the puromycin-insensitive fraction of polymerase activity in exponential or stationary phase

cultures. Rather, the data are consistent with the concept that the synthesis of a certain fraction of RNA, possibly ribosomal, utilizes a critical protein(s) which must be continuously resupplied. The limited availability of this protein then curtails the polymerase activity concerned with the synthesis of this fraction of RNA.

Evidence for this concept can be found in the data of Holland (1963) and Tamaoki and Mueller (1965), who showed differential effects of puromycin on the synthesis of different classes of RNA in HeLa. Holland found that concentrations of puromycin which greatly inhibited ribosomal RNA synthesis had considerably less effect on the synthesis of soluble and messenger RNA fractions. Nakada (1965; Nakada and Marquisee, 1964) has suggested that stringent strains of Escherichia coli use a particular ribosomal protein to combine with ribosomal RNA as it is synthesized and remove it from the DNA template, thus promoting the further synthesis of ribosomal RNA.

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