A STUDY OF FACTORS REGULATING RNA SYNTHESIS IN HELA CELLS USING MPB, A REVERSIBLE INHIBITOR OF RNA SYNTHESIS[1]

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2-Mercapto-1-(β-4-pyridethyl)benzimidazole (MPB) was recently shown to inhibit nucleic acid synthesis strikingly in a number of mammalian cells while affecting protein synthesis only minimally (Bucknall and Carter, 1967). The agent is particularly useful for temporary limitations of RNA synthesis since its action in living cells is readily reversed on medium change. The present experiments show that nuclei isolated from MPB treated HeLa cells also resume RNA synthesis when supplied with the four ribonucleoside triphosphates. The RNA polymerase activity of such nuclei is equal to that of nuclei from control cells even though RNA synthesis had been blocked for extended periods. This agent has also been found to protect cells against the loss of RNA polymerase activity which attends the inhibition of protein synthesis by cycloheximide or puromycin (Summers, Noteboom, and Mueller, 1966). Accordingly MPB provides a useful tool for studying factors which regulate RNA polymerase activity in living cells.

Methods. HeLa cells were maintained in exponential growth in suspension cultures in a modified Eagle's medium (Mueller, et al., 1962). Nuclei were isolated by hypotonic treatment and assayed for RNA polymerase as previously

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described (Summers, et al., 1966). Final concentrations of reagents in the 1 ml reaction mixture were 0.1M Tris (pH 8.0), 0.075M KCl, 0.005M $MgCl_2$, 0.03M NaF, 0.01M cysteine, 10^{-3}M ATP; $4x10^{-4}$M each GTP and UTP, and 10^{-6}M CTP-H^3 at a specific activity of 1C/mmole. Nuclei from $4x10^6$ cells were routinely incubated in each sample. The RNA synthesis at 37° was terminated by rapid chilling and precipitation of the product with 4% perchloric acid (PCA) in the presence of serum protein as carrier. The acid-washed precipitates were dissolved in formic acid and the radioactivity determined by liquid scintillation counting.

Rates of protein synthesis in intact cells ("in vivo") were compared by 30-minute incubations of aliquots of the cell cultures with H^3-L-leucine and measurement of the PCA-insoluble counts at the end of the labeling period. RNA synthesis rates in intact cells were measured from the incorporation of H^3-cytidine over 30 minute intervals. After precipitation of the cells with PCA, the radioactivity released into the acid soluble fraction with ribonuclease digestion was determined. These procedures were described previously (Summers, et al., 1966).

The MPB was dissolved in dimethyl sulfoxide (DMSO) and one ml added per 1000 ml of medium; the final MPB concentration was 25 μg per ml. Various levels of the compound were added also directly to the 1 ml polymerase assay system in 10 μl DMSO. Puromycin or cycloheximide was added to the cell cultures for a final concentration of 20 μg per ml.

Results and Discussion. MPB rapidly inhibited more than 90% of the total in vivo RNA synthesis in cells in exponential growth, while protein synthesis decreased more slowly in response to the inhibitor (Figure 1). DNA synthesis studied in synchronized cells was not affected by MPB once this process was initiated (Kajiwara, et al.). The same concentration of DMSO alone had no inhibitory effect on RNA or protein synthesis.

Whereas RNA synthesis was inhibited in intact cells, nuclei from cells pretreated with MPB for 0 to 60 minutes exhibited RNA polymerase activity

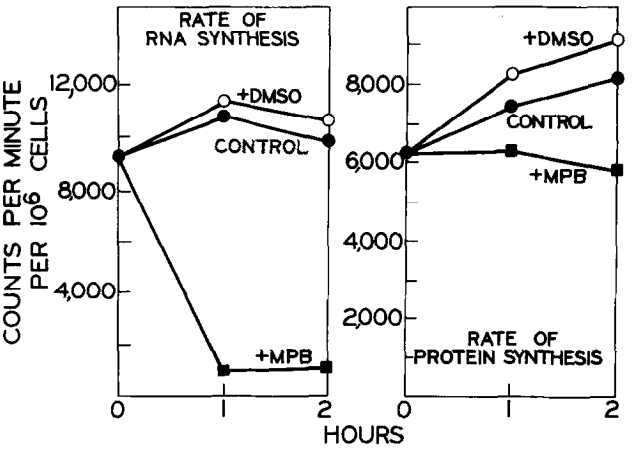

Fig. 1. The effect of MPB on rates of RNA and protein synthesis in vivo. Additions at 0 time were 0.001 ml DMSO (+DMSO) or 25 μg MPB in 0.001 ml DMSO (+MPB) per ml cell culture. RNA synthesis was measured as acid insoluble, RNase-releasable counts from 30 min pulse with H^3-cytidine (0.25 μC/ml, 50 mC/mmole), protein synthesis as acid insoluble counts after 30 min, labeling with H^3-L-leucine (0.4 μC/ml, 5C/mmole).

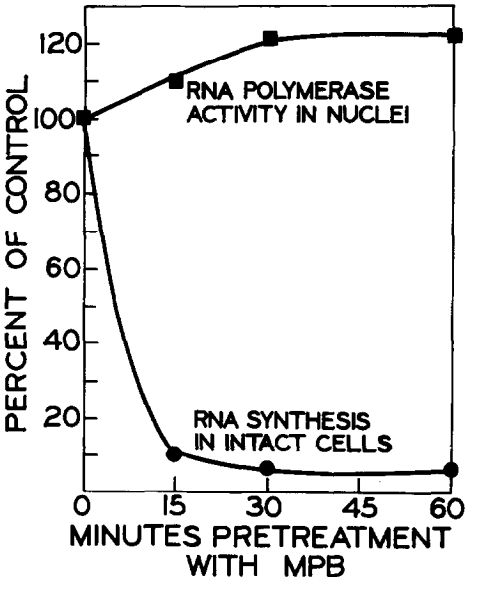

Fig. 2. The effect of MPB on rate of RNA synthesis in intact cells and the level of RNA polymerase activity. MPB (25 μg/ml) was added at 0 time and in vivo RNA synthesis measured as in Fig. 1. At each point nuclei were isolated and RNA polymerase activity assayed by a 30 min incorporation of H^3-CTP to acid-insoluble counts.

Table 1. The effect of actinomycin D (0.1 μg/ml) on rate of RNA synthesis in intact cells and on nuclear RNA polymerase activity. H^3-cytidine incorporation *in vivo* and H^3-CTP incorporation by the isolated nuclei were determined after actinomycin addition to the cells in culture in an experiment similar to that in Fig. 2.

Minutes Act. D Preincubation	Rate of RNA Synthesis *in vivo* (% of control)	Nuclear RNA Polymerase Activity (% of control)
0	100	100
30	43	54
60	41	47

Table 2. The inability of MPB to inhibit nuclear RNA polymerase activity directly. Nuclei from untreated cells were incubated for 30 min in the standard 1 ml assay system with indicated amounts of MPB added directly in 10 μl DMSO to the assay system.

	μg MPB/ml	CPM/4 x 10^6	% of Control
Expt. 1	0	2320	100
	10	2220	96
	50	2550	110
	100	2370	102
	DMSO only	2500	108
Expt. 2	0	2620	100
	250	2410	93
	DMSO only	2670	102

near the control level (Figure 2). This result is different from that of the analogous experiment with actinomycin D (Table 1), in which cells inhibited with a low concentration of actinomycin *in vivo* yielded nuclei with proportionally decreased capacity for RNA synthesis *in vitro*. MPB also had no direct effect on incorporation of H^3-CTP into RNA when included in concentrations up to 250 μg per ml in the isolated nuclei polymerase assay (Table 2). These results illustrate that RNA polymerase can survive for significant intervals in cells in which RNA synthesis has been inhibited

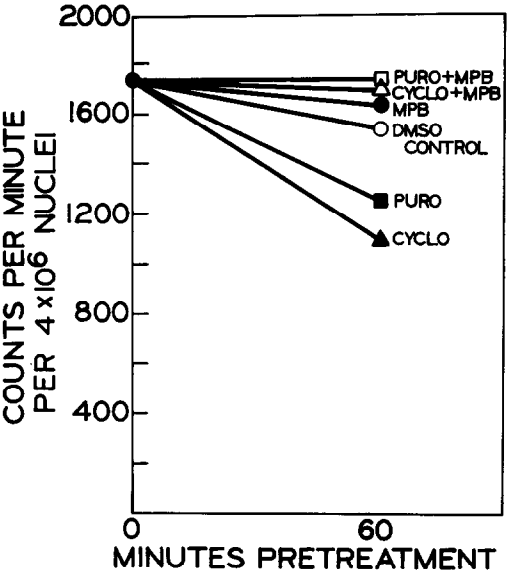

Fig. 3. RNA polymerase activity of nuclei isolated after one hour pretreatment of the cells in culture with 20 μg per ml puromycin or cycloheximide $\pm$ 25 μg/ml MPB or with MPB only. The same amount of DMSO was added to each culture. Polymerase activity was measured by incorporation of H^3-CTP over a 30 minute period.

profoundly and thus MPB can be used to study cellular processes which regulate the level of RNA polymerase activity independent of RNA synthesis itself.

Previous work in this laboratory showed that inhibition of protein synthesis in the intact cells was attended by a striking reduction of RNA synthesis and that isolated nuclei from such cells had decreased RNA polymerase activity (Summers, et al., 1966). Since protein synthesis was only minimally affected during MPB treatment this agent afforded a method to study the relationship of protein synthesis to RNA synthesis. For this purpose identical cultures of cells were pretreated for 1 hour with puromycin or cycloheximide in the presence and absence of MPB. The nuclei were then isolated and assayed for RNA polymerase activity. In accord with the earlier study both puromycin and cycloheximide reduced the polymerase activity (with DMSO included as a control); however, the co-addition of

Table 3. Rates of RNA and protein synthesis *in vivo* and nuclear RNA polymerase activity of cells preincubated 1 hour with puromycin or cycloheximide ± MPB. Incorporation of H^3-cytidine or H^3-L-leucine in 30 min pulses as in Fig. 1 were used to compare *in vivo* RNA and protein synthesis in the experiment described in Fig. 3.

Pretreatment	RNA Polymerase	*in vivo* RNA	*in vivo* protein
	% of untreated controls		
Control	100%	100%	100%
DMSO only	97	98	96
MPB in DMSO	99	6	64
Puromycin + DMSO	73	59	20
Cycloheximide + DMSO	64	69	4
Puromycin + MPB + DMSO	98	6	4
Cycloheximide + MPB + DMSO	98	8	3

MPB (in DMSO) completely protected against this loss of nuclear activity (Fig. 3). Table 3 summarizes the RNA polymerase data along with the relative rates of *in vivo* RNA and protein synthesis in the same cultures.

These results show that the protein synthesis inhibitors did not affect polymerase activity unless RNA synthesis was permitted to continue. This finding is in accord with the utilization of a critical, rate-limiting protein during the native process of RNA synthesis in mammalian cells. Previous studies from this laboratory have shown that this dependency can be quite acute under conditions of induced growth (Nicolette and Mueller, 1966).

MPB has proven useful in delineating intermediate steps in the synthesis of RNA in mammalian cells and in the temporal disturbance of RNA synthesis in such sequential processes as the cell cycle. These studies will be reported elsewhere.

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