SELECTIVE INHIBITION OF PLASMID DNA PRODUCTION IN BACILLUS MEGATERIUM BY 6-(p-HYDROXY-PHENYLAZO)-URACIL: EVIDENCE FOR MULTIPLE MAINTENANCE SYSTEMS

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SUMMARY: The substituted pyrimidine analog 6-(p-hydroxy-phenylazo)-uracil, a specific inhibitor of DNA replication in Gram-positive bacteria, inhibits incorporation of radioactive nucleotides into DNA, but not RNA, of Bacillus megaterium at sub-bacteriocidal levels. Incorporation into chromosomal DNA is much more severely inhibited than into the polydisperse circular DNA of unknown function produced by the organism. The effect on the circular fraction appears to be primarily on the larger molecules, since incorporation into the two smallest plasmids appears insensitive to the drug. These results suggest that at least two maintenance systems are involved in the production of this complex array of plasmid molecules.

We have previously described the existence and some of the physical properties of a polydisperse array of circular duplex DNA molecules produced in exceptionally high quantities by logarithmically-dividing cells of the Gram-positive sporeforming bacterium, Bacillus megaterium (1,2). These molecules have been shown to fall into a minimum of eight discrete size classes by electron microscopic and sedimentation velocity analyses (3). The molecular size distributions range from 3.9 million daltons for the smallest molecules to nearly 100 million daltons for the largest sizes (3). The rather unique properties of this system, particularly the highly disperse size distribution and the production of multiple copies of several different size molecules per cell has prompted an examination of their mode of replication and maintenance in the dividing cells. One approach to this objective involves the use of a metabolic inhibitor of known function, HPUra[1]. HPUra, an azo pyrimidine drug, has been shown to affect DNA synthesis by a specific inhibition of chromosome replication in Gram-positive

[1]HPUra: 6-(p-hydroxy-phenylazo)-uracil.

bacteria (4-7). The compound has little or no effect on *in vivo* phage DNA replication (8) or repair synthesis (9) in *Bacillus subtilis*, or on any of the known DNA polymerases of *E. coli* (7). I report here the effects of HPUra on the maintenance of the circular DNA molecules in *Bacillus megaterium*.

MATERIALS AND METHODS

Growth and labelling conditions - *B. megaterium* strain 216 was grown in Spizizen's minimal medium (10) supplemented with 0.02% Difco yeast extract and 0.4% glucose. For circular and chromosomal DNA labelling comparisons, cultures in mid-log phase were divided into 5 ml aliquots in 50 ml side-arm Erlenmeyer flasks, and 10 µCi of [^{3}H]-thymidine (sp. act. 22 mCi/M mole) were added simultaneously with or without various concentrations of inhibitor. The oxidized form of HPUra was added directly from a 50 mM stock solution in 0.05 N NaOH. The reduced form of the drug was prepared by preincubation of the oxidized stock solution in 50 mM dithiothreitol at 37° for 20 min prior to use. After 20 minutes sodium azide was added (50 mM final concentration) to kill the cells, which were then chilled on ice, washed twice with 5 ml of cold TES[2] buffer, and lysed by a lysozyme and Sarkosyl treatment (1). Aliquots of the crude lysate were spotted on Whatman 3 MM filter paper disks for determination of total [^{3}H]-thymidine incorporation into acid-insoluble DNA (11). Samples of identical sizes were then centrifuged to equilibrium in cesium chloride-ethidium bromide gradients to separate the circular DNA from the linear (chromosomal) material, as described previously (1). In one experiment where greater amounts of DNA were desired for further examination, 500 ml cultures were pre-treated for 15 minutes with HPUra prior to addition of [^{3}H]-thymidine. Incubation was then continued for 1 hr prior to processing of the cells. After dye-buoyant density centrifugation, fractions containing circular DNA and chromosomal DNA were pooled separately, freed of dye and

[2]TES: Tris-hydroxymethyl aminomethane hydrochloride, 30 mM; ethylenediamine tetraacetic acid, 6 mM; sodium chloride, 50 mM; pH 8.0.

CsCl, and concentrated for sedimentation velocity analysis by a previously described procedure (3).

For DNA and RNA labelling comparisons, mid-log phase cultures were divided into 20 ml aliquots in 250 ml side-arm Erlenmeyer flasks, and 16 μCi (sp. act. 1.58 mCi/M mole) of [^{14}C]-adenine (Mallinckrodt) were added with or without the inhibitor. At various intervals, four 0.5 ml aliquots were withdrawn and mixed with equal volumes of ice cold 20% TCA[3]. Two ml of cold 10% TCA were then added and the samples allowed to stand on ice for at least 30 minutes. DNA and RNA were estimated by a modification of the procedures described by Kennell (12). The TCA-insoluble precipitates were first collected on Whatman 3 MM filter paper disks and rinsed with two 2 ml portions of cold 10% TCA. After drying, two samples of each time set were digested for 2 hr with 2 ml of 1 N NaOH at 37°. The filters were then placed between layers of Whatman #1 filter paper on a 15 cm Buchner funnel (maximum of 6 layers per batch) and washed successively with cold 10% TCA (3X), cold 70% ethanol (1X), 70% ethanol at 40° (2X), 95% ethanol - diethyl ether (1:1) at 40° (2X), and finally with diethyl ether (room temp). The dried filters were counted in a PPO[4]/toluene mixture (2.5 g PPO/liter) in a Beckman liquid scintillation spectrometer. [^{3}H] counting efficiency was 32% and [^{14}C] efficiency was 57%. The DNA incorporation values were taken as the average numbers of counts remaining in the alkali-hydrolyzed samples, and the RNA incorporation values were taken as the differences between the DNA values and the average numbers of counts in the unhydrolyzed samples.

RESULTS AND DISCUSSION:

Under the conditions employed in these experiments, HPUra produced about 50% inhibition of growth at concentrations of 8-10 μM in the medium. The onset of growth inhibition appeared after about 2-1/2 hours following addition

[3]TCA: Trichloroacetic acid.

[4]PPO: 2,5-Diphenyloxazole.

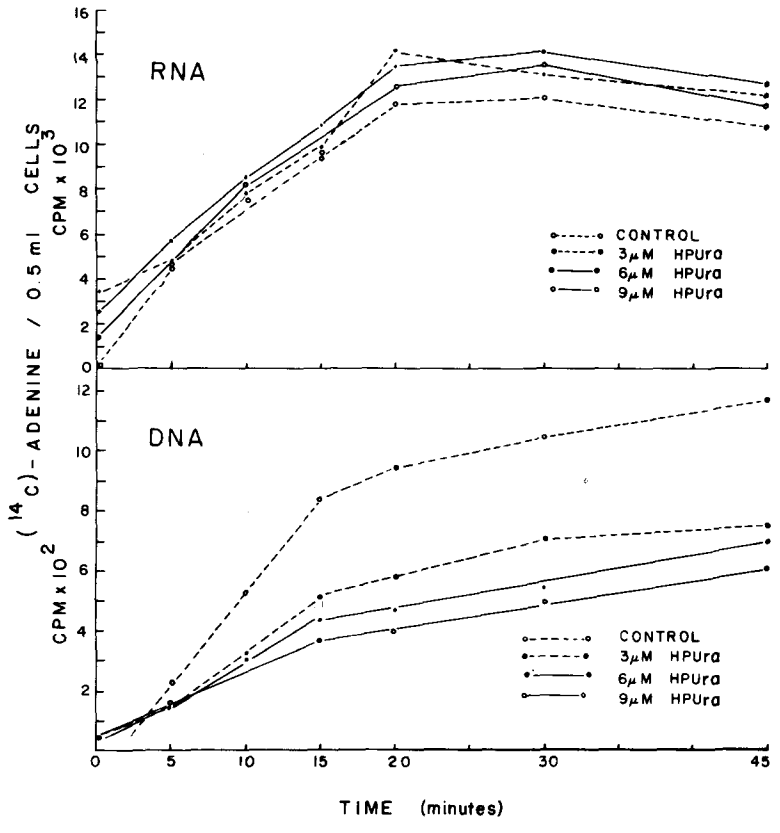

Fig. 1: Effects of HPUra on incorporation of adenine into alkali-labile (RNA) and alkali-stable (DNA) nucleic acids.

A culture of B. megaterium in mid-logarithmic phase was divided into four equal portions and one sample was exposed to 0.8 μCi/ml of [^{14}C]-adenine while the others received [^{14}C]-adenine plus HPUra (oxidized form) to the indicated final concentration. The cultures were incubated at 37° with shaking and aliquots of 0.5 ml were removed at intervals for determination of incorporation of [^{14}C] into DNA and RNA as described in Materials and Methods.

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of the drug -- prior to this time the growth rate was essentially identical to that of the control culture. This finding is similar to that originally made by Brown and Handschumacher (4) with Streptococcus fecalis which suggested that the drug induces a state of unbalanced growth as a secondary effect of inhibition of chromosome replication, analogous to the phenomenon of thymineless death (13). The specificity of the inhibitory effect of HPUra on DNA synthesis and not on RNA synthesis is shown by the experiment in Figure 1, which compares the kinetics of [^{14}C]-adenine incorporation into alkali-stable

TABLE I

Effects of HPUra on Incorporation of Thymidine into DNA of *Bacillus megaterium*

Treatment	% Relative Total Incorp.*	% Relative Incorp.** into Fractionated			% in Circular++
		Circular	Chromosomal	Total+	
Control (No HPUra)	100	100	100	100	26
Reduced HPUra					
10 µM	29	53	22	30	45
20 µM	19	25	11	15	44
50 µM	8	17	4.5	8	58
Oxidized HPUra					
10 µM	5	16	4.4	7.6	56
20 µM	3.4	9	1.9	3.9	62
50 µM	0.8	1.3	0.3	0.6	--

*Determined as TCA-insoluble radioactivity in crude cell lysates prepared from identical volumes of culture.

**Based on total radioactivity in DNA recovered from uniform size samples centrifuged in CsCl-ethidium bromide equilibrium gradients.

+Summation of radioactivity recovered from both fractions, expressed as percentage of control sample.

++Proportion of total radioactivity recovered from gradients as circular DNA.

(DNA) and alkali-labile (RNA) nucleic acids. It is evident that concentrations of HPUra which yielded substantial inhibition of incorporation into DNA did not decrease RNA labelling. Similar experiments not presented here (Carlton, B. C., unpublished) with Actinomycin D and rifampin, known inhibitors of RNA synthesis, led to the inverse result (i.e., incorporation into alkali-labile material was severely inhibited).

The effects of the drug on incorporation of labelled thymidine into DNA are shown in Table 1. Concentrations of the drug sufficient to cause growth inhibition after 2-3 hrs brought about substantial inhibition of thymidine

incorporation into total DNA during a 20 minute pulse experiment (column 1). When the reduced and oxidized forms of the drug were compared it was found that the reduced form was approximately 5 times less effective than the oxidized form in inhibiting thymidine incorporation. This observation is perhaps surprising, since Neville and Brown (5), Bazill and Gross (6), and Gass et al. (7) have shown that only the reduced form of HPUra is effective in in vitro inhibition of DNA polymerase. This finding is, however, consistent with the in vivo results obtained initially by Brown and Handschumacher (4) with S. fecalis. The explanation for this apparent paradox is not known, although it may be that the cells are impermeable to the reduced (active) form of the drug, thereby leading to lower intracellular concentrations than when the inactive oxidized form is used. The latter, however, once inside the cell, may then be reduced to the active inhibitory form.

A further examination of the circular and chromosomal DNA fractions recovered from dye-buoyant density gradients (Table 1) reveals that the incorporation of thymidine into both fractions was inhibited by the drug. The extent of inhibition was much greater on the chromosomal fraction than on the circular (plasmid) fraction, however. For example, whereas oxidized HPUra at 10 μM final concentration inhibited thymidine incorporation into chromosomal DNA by about 95%, the inhibition of circular DNA labelling was only 84%. That the different inhibitory effects are not due to differential recoveries of the two DNA fractions during the isolation or centrifugation procedure is suggested by the data in column 4. As shown here, when the total radioactivity from both the circular and chromosomal DNA fractions is determined, the recoveries relative to the control samples are in good agreement with the relative amounts of label originally determined in the crude lysates (column 1). The last column in Table 1 shows the total per cent of labelled thymidine recovered in circular DNA for each of the HPUra levels. When compared to the control, there is an enrichment of about two-fold observed for the circular DNA in the HPUra-inhibited cultures although this enrichment does not exceed

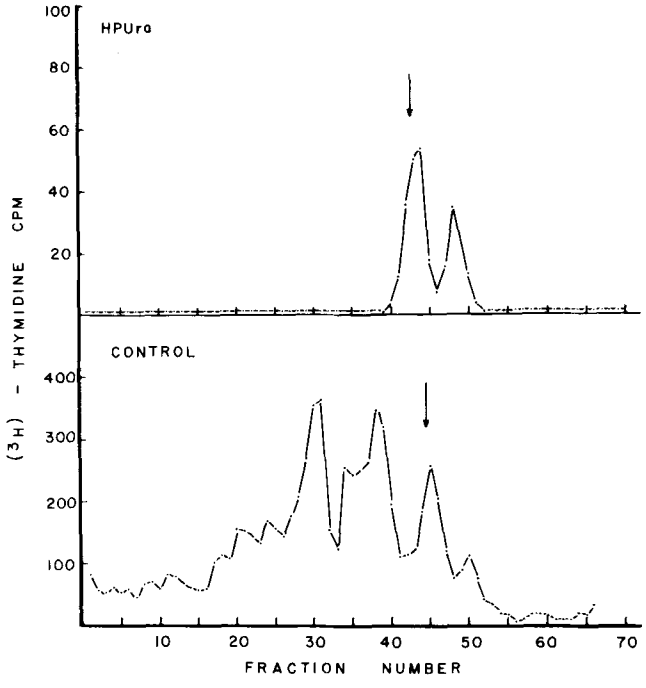

Fig. 2: Sedimentation velocity profiles of the circular DNA from HPUra-treated and control cultures of B. megaterium.

One 500 ml culture of B. megaterium in the early-logarithmic phase of growth (ca. 5 x 10^6 cells/ml) was treated with HPUra (oxidized) to 8 μM final concentration. After 15 minutes exposure to the drug 100 μCi of [^{3}H]-thymidine (sp. act. 22 mCi/mM) were added to the drug-treated culture and to a companion control culture. Incubation on a 37° rotary shaker was continued for one hour in which time both cultures attained a cell density of 2-3 x 10^7 cells/ml. DNA from both cultures was then isolated and the circular and chromosomal components fractionated by preparative dye-buoyant-density procedures (1). Aliquots of the [^{3}H]-labelled circular DNA fraction from the control and HPUra-treated cultures were mixed with [^{14}C]-labelled T7 phage DNA marker and centrifuged through neutral 5-20% sucrose gradients and assayed as described previously (3). The direction of centrifugation was from right to left in the profiles. The arrows indicate the position of the T7 phage DNA marker. Radioactivity was corrected for background.

a level of about 60-65% of the total DNA. Clearly, the drug inhibits not only chromosomal DNA production but also, to a lesser extent, circular DNA as well.

In order to test whether the inhibitory effect on thymidine incorporation into the plasmid DNA occurred with all size fractions, samples of the circular DNA recovered from CsCl-ethidium bromide gradients of control and drug-inhibited cultures were centrifuged through 5 to 20% neutral sucrose gradients.

The results (Figure 2) show that the HPUra had a differential inhibitory effect on the larger (faster sedimenting) circular DNA molecules. All of the label in drug-inhibited cultures was found in the two smallest plasmid species, shown previously to have molecular weights of 3.9 and 6.2 million (3). It is thus concluded that the HPUra inhibits differentially the incorporation of thymidine into larger plasmid molecules while there is little or no inhibition of incorporation into the two smallest species. These results have been further confirmed by growing the organism in sub-lethal concentrations of HPUra for several generations and comparing the distribution of circular DNA bands observed after electrophoresis on agarose gels (not shown). Again, the two smallest circular DNA fractions were retained while the larger ones appeared to be lost, suggesting that the HPUra interferes principally with maintenance of the larger plasmids. One point of caution that should be noted in these interpretations is the possibility that the drug does not specifically inhibit synthesis of the larger circular DNA forms but only their final processing into covalent circular duplexes. Were this to occur, the potential plasmid molecules would be recovered in the chromosomal band instead of the denser, covalently closed circular band during the CsCl-ethidium bromide fractionation. Although such an effect of the drug cannot be completely ruled out, it seems unlikely based on agarose gel patterns and sedimentation velocity profiles of the chromosomal DNA band obtained from drug-inhibited cultures.

The results of these experiments with this azopyrimidine drug are interpreted as indicating that at least two maintenance mechanisms exist for the circular DNA plasmids of unknown function in B. megaterium. One of these, presumably responsible for replication of the chromosome and for maintenance of the plasmids of molecular weight 16 million and above, is inhibited by the drug. Another, which maintains the 3.9 and 6.2 million plasmids, is insensitive to the drug. This postulated dichotomy in plasmid maintenance mechanisms is in some ways analogous to the differential maintenance requirements for

various plasmids observed in Escherichia coli. The colicinogenic plasmid E_1 is dependent upon DNA polymerase I for its maintenance (14), while col V, col Ib, two R factors, and an F element are apparently under the control of DNA polymerase III (15). Whether analogous enzymatic functions can be assigned to the maintenance mechanisms involved in the B. megaterium plasmid system must await the isolation and characterization of the DNA replication enzyme(s) in this organism.

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