Inhibition of mammalian RNA polymerase by 5,6-dichlororibofuranosylbenzimidazole (DRB) and DRB triphosphate

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ABSTRACT

DRB triphosphate inhibits activity of isolated RNA polymerase B, and, to a lesser extent, that of polymerase A. The same holds true for transcription in isolated nuclei. It does not act as an initiation inhibitor. In all cases, high concentrations of DRB triphosphate are required. Cells do not phosphorylate DRB to a measurable extent. hn RNA resistant to DRB is initiated with both ATP and GTP in the presence of the drug. These experiments render the hypothesis unlikely that DRB triphosphate in the cell specifically interferes with the initiation reaction of polymerase B.

INTRODUCTION

The nucleoside analogue 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) has recently received much attention as an inhibitor of RNA synthesis in eukaryotic cells (1). It inhibits hn RNA synthesis partially, whereas m-RNA production is suppressed by more than 95 % (2-4). Only at higher concentrations are r-RNA synthesis and processing affected (5).

On the basis of pulse-chase experiments, it has been concluded that the drug acts as an inhibitor of hn RNA chain initiation (6-7). We recently demonstrated that pretreatment of Ehrlich ascites cell cultures with the drug results in an elimination of a specific fraction of RNA polymerase B active in the isolated nuclei (8). Polymerase A did not respond to this drug treatment.

The action of DRB on cell metabolism has recently been extensively reviewed by Sehgal and Tamm (9). In a comment on the effect on RNA metabolism, the authors pose the question for future work: "Is DRB itself the active component or is it metabolized by the cell to an active derivative such as triphosphate?". The present paper deals with this question.

MATERIALS AND METHODS

Synthesis of DRB triphosphate. DRB triphosphate was synthesized as described in detail elsewhere (10). DRB monophosphate was a generous gift of Dr. J. Guglielmi. This was converted to the triphosphate by the method of Hoard & Ott (11). The structure of the triphosphate was confirmed by a) a limited digest with alkaline phosphatase, b) conversion to the diphosphate by treatment with the S1 fragment of myosin, c) conversion to the monophosphate by phosphodiesterase. Moreover, this chemically synthesized DRB triphosphate co-chromatographed in several thin-layer chromatography systems with the major product that resulted from an enzymatic phosphorylation of DRB monophosphate with [γ - 32 P] ATP and myokinase.

RNA polymerase. Polymerase A was prepared from Ehrlich ascites cells as described by Roeder & Rutter (12).

Polymerase B was prepared from calf thymus as described (13). RNA polymerase from *E.coli* (holoenzyme) was a gift of Dr. H. Blüthmann.

All other materials were as previously described (8, 14).

RESULTS

Effect of DRB triphosphates on isolated polymerases. The activity of mammalian RNA polymerases A and B and of the *E.coli* holoenzyme was monitored by using poly[d(AT)] and poly[d(GC)] as templates. The assays were incubated with increasing amounts of DRB triphosphate. As demonstrated in Fig. 1, all polymerases are inhibited by the agent. Polymerase B was 5 to 6fold more sensitive than polymerase A and *E.coli* RNA polymerase. Inhibition of polymerase B was more pronounced with poly[d(AT)] as template as compared to poly[d(GC)]. In all cases, the inhibitor concentration required for 50 % inhibition greatly exceeds the concentration of the nucleoside triphosphates used as substrates. The requirement for this high concentration of DRB triphosphate raises the possibility that the inhibitory effect was due to a perturbation of the free divalent cation concentration in the assays. This has been excluded by control experiments: The inhibitory effect of DRB triphosphate is not influenced by higher concentrations of $MnCl_2$ or $MgCl_2$ (data not shown). Comparison of Fig. 1a and 1c reveals that the extent of inhibition is dependent on the molar ratio of inhibitor to substrate.

Does DRB triphosphate inhibit the initiation reaction? An inhibitor specific for the initiation reaction should allow elongation of RNA chains when given after initiation has been allowed to occur in the absence of the inhibitor. This approach was used for the experiments described in Fig. 2.

Figure 1: Inhibition of isolated RNA polymerase by DRB triphosphate.

Transcription assays were performed as described (14) in the presence of the indicated amounts of DRB triphosphate. The assays contained in a volume of 50 μ l: a) 0.1 unit polymerase B from calf thymus, and b) 0.02 unit polymerase A from Ehrlich ascites cells; ATP and UTP 0.02 mM each, and 1 μ g poly[d(AT)] (x) or GTP and CTP 0.02 mM each, and 1 μ g poly[d(GC)] (●) as template. Assays for polymerase A contained 4 mM $MgCl_2$ instead of $MnCl_2$, and no KCl.

c) 1 unit *E. coli* holoenzyme (●), and 0.1 unit polymerase B (x), ATP and UTP 0.2 mM each, and 1 μ g of poly[d(AT)] per test. Assays for *E. coli* holoenzyme contained 30 mM Tris-HCl, pH 7.8, 30 mM $MgCl_2$, 130 mM $(NH_4)Cl$, and 2 mM dithiothreitol.

No significant difference in RNA polymerase activity was observed when the inhibitor was added before or after the initiation reaction. The validity of this experimental approach was verified by using aurintricarboxylic acid (ATA) as inhibitor (Fig. 2b) (ATA is known to interfere with the binding of polymerase to DNA but allows continuation of preinitiated RNA chains (15)). In this case, a clear difference in the amount of RNA synthesized between the two assay conditions is obvious.

In a second experiment initiations of new chains were monitored by the simultaneous incorporation of $[\gamma\text{-P}^{32}]$ and $[^3\text{H}]$ ATP into RNA (Fig. 3). An instantaneous stop of incorporation of both isotopes was observed after addition of DRB triphosphate. If DRB triphosphate were an inhibitor specific for

Figure 2: Effects of DRB triphosphate and of aurintricarboxylic acid on the initiation reaction of RNA polymerase B. Transcription assays containing RNA polymerase B, 1 μg poly[d(AT)], and ATP and GTP 0.02 mM each, were preincubated for 2 min at 37°C in the presence (●) or absence (x) of the amounts of inhibitor as indicated. Then [³H]UTP (2.5 $\mu\text{Ci}/\text{assay}$) (●) or [³H]UTP and inhibitor (x) were added and incubation was continued for 2 min. a) DRB triphosphate; b) aurintricarboxylic acid as inhibitor.

the initiation reaction incorporation of [^3H]AMP should have continued for at least 2 min (see Fig. 2b and Ref. 8) in contrast to the [$\gamma\text{-}^{32}\text{P}$] ATP incorporation.

Effect of DRB triphosphate on RNA synthesis in isolated nuclei. Fig. 4 shows that polymerase B in nuclei isolated by lysis of cells with saponin (8) was more sensitive to DRB triphosphate than polymerase A, as was found with the isolated enzymes. The differences of the dose-effect curves may be explained by the higher substrate concentrations used in the assays of the isolated nuclei.

Additional experiments (data not shown) revealed that neither DRB nor DRB monophosphate have any effect on transcription in either the isolated nuclei or the purified polymerase systems (26).

Is DRB phosphorylated by the cells? The use of DRB monophosphate and DRB triphosphate as reference substances in chromatographic experiments allowed us to study whether DRB is phosphorylated *in vivo*. Cells were incubated in the presence of DRB with labeled phosphate. Acid soluble material was extracted and subjected to two-dimensional thin-layer chromatography. As controls, extracts were prepared from cells labeled in the absence of DRB or in

Figure 3: Kinetics of [$\gamma\text{-}^{32}\text{P}$] ATP and [^3H] AMP incorporation after addition of DRB triphosphate. 2 ml assay reactions containing polymerase B, 0.05 μg poly[d(AT)], 0.02 mM UTP, 0.02 mM [$\gamma\text{-}^{32}\text{P}$] ATP (2.5×10^4 cpm/pmol), and [^3H] ATP (175 cpm/pmol) were incubated at 37°C. At the times indicated aliquots of 100 μl were removed and precipitated with TCA. After 2.5 min, an aliquot was removed and brought to a final concentration of 2 mM DRB triphosphate (arrow). (●) TCA precipitable material of control, (x) of samples containing DRB triphosphate. a) [$\gamma\text{-}^{32}\text{P}$] ATP incorporation; b) [^3H] AMP incorporation.

Figure 4: Inhibition of transcription by DRB triphosphate in saponin-lysed Ehrlich ascites cells. Preparation of saponin-lysed cells and assay for transcription were as described (8), with the amounts of DRB triphosphate as indicated. Assay without (●) or with (x) 1 µg/ml α-Amanitin (from Boehringer).

the presence of 3'-deoxyadenosine (Cordycepin). The conversion of 3'-deoxyadenosine to its triphosphate was readily observed by the appearance of label at about the position of 2'dATP. No radioactive material comigrated with the DRB triphosphate or DRB monophosphate markers (Fig. 5). Prolonged exposure of the chromatograms as well as excision of the corresponding spots and quantitation of the radioactivity allows us to conclude that less than 0.01 % of the amount of label found in GTP or ATP occurs in DRB triphosphate (data not shown).

Initiations of hn RNA in the presence of DRB. It is known that newly synthesized hn RNA molecules carry 5' triphosphate ends, 70 % being GTP and 30 % ATP (18-21). Since hn RNA is only partially inhibited by DRB the possibility exists that either GTP or ATP initiations are specifically affected. In this case, hn RNA synthesized in the presence of DRB should carry only one of the 5' purine triphosphate ends. This hypothesis was tested by application of the method of tetrphosphate analysis as previously described (8, 18). Cells were labeled with [³²P]phosphate for 30 min in presence or absence of DRB. Low concentrations of actinomycin were applied to the cultures in order to eliminate the labeling of r-RNA (21). RNA larger than 18 S was isolated and analysed for pppGp and pppAp content. The final step of this analysis is shown in Fig. 6. Both nucleoside tetrphosphates were found in the hn RNA

Figure 5: Two-dimensional thin-layer chromatography of acid-soluble material from cells ^{32}P -labeled in the presence of nucleoside analogues. Ehrlich ascites cells (5×10^6 cells in 0.5 ml medium) were labeled with $12.5 \mu\text{Ci}$ [^{32}P]phosphate for 30 min with a) $50 \mu\text{g}$ DRB in $10 \mu\text{l}$ 50% DMSO or b) $50 \mu\text{g}$ 3'deoxyadenosine in $10 \mu\text{l}$ 50% DMSO or c) with $10 \mu\text{l}$ 50% DMSO. The cells were then washed with an excess of cold phosphate-buffered saline and extracted for 15 min at 0°C with $20 \mu\text{l}$ 10% perchloric acid containing all four ribonucleoside triphosphates as OD markers. Extraction was repeated once with 3% PCA. The extracts were treated with charcoal and the nucleotides extracted from the charcoal essentially as described by Gonzales and Geel (16). The extracts were subjected to two-dimensional chromatography on poly(ethylene)imine cellulose thin layers (Cel 300 PEI UV 254 from Machery & Nagel) as described by Cashel et al. (17), except that 1 M phosphate was used as eluant in the second dimension. DRB monophosphate and DRB triphosphate were added to sample a) as OD markers. ^{32}P -labeled materials were visualized by exposing X-ray film to the dried chromatograms for 15 h (shaded areas) and identified by superposition on the OD markers (closed circles). There was no essential difference in the pattern of labeled nucleotides when the purification by adsorption to charcoal was omitted.

fractions labeled in the presence of DRB. It was confirmed in chromatographic experiments like that shown in Fig. 5 that neither DRB nor actinomycin impaired the uptake of ^{32}P -label into all four nucleoside triphosphates (data not shown).

Figure 6: 5' endgroup analysis of hn RNA ^{32}P -labeled in the presence or absence of DRB. Ehrlich ascites cells ($1 \times 10^7/\text{ml}$ in Eagles medium lacking inorganic phosphate) were preincubated in the presence of $80 \mu\text{g}/\text{ml}$ actinomycin D and with or without $50 \mu\text{g}/\text{ml}$ DRB for 30 min. Each culture was then labeled with $1 \text{ mCi}/\text{ml}$ [^{32}P] phosphate for 30 min. The cells were washed once with cold phosphate-buffered saline, and RNA was extracted as described by Kwan et al. (22). Low molecular weight material was removed and RNA $> 18 \text{ s}$ was isolated as described (8). This was digested with RNase T_2 after addition of [^3H] pppAp (2000 cpm) and [^3H] pppGp (5000 cpm) as markers. [^{32}P] pppAp and [^{32}P] pppGp were purified from the digests using DEAE-Sephadex A25 capillary columns (8, 18) and rechromatographed on a second capillary column: a) pppAp and b) pppGp from control cells; c) pppAp and d) pppGp from DRB-treated cells. The pppAp preparations contain a shoulder of pppGp.

DISCUSSION

The mechanism by which DRB interferes with the synthesis of hn RNA is still unknown. Since the drug is a nucleoside analogue, it seemed likely that DRB is phosphorylated upon entry into the cell and exerts its effect at the triphosphate level (1, 9). The specific effect on RNA polymerase B could be explained by a higher affinity of this enzyme for DRB triphosphate as compared to polymerase A and C. A preferential inhibition of the initiation reaction could be due to the more stringent requirements of this step as compared to elongation (23, 24). Moreover, the finding that only a fraction of hn RNA is sensitive to the drug could be explained by a heterogeneity of the initiation event; possibly only GTP or ATP initiations are inhibited.

Our initial findings that DRB triphosphate inhibits the transcriptional activity of isolated RNA polymerase and of isolated nuclei are consistent with this hypothesis (Figs. 1 and 4). These experiments also revealed the preferential inhibition of polymerase B in these systems in analogy to the effect of DRB in vivo. The fact that the inhibitory action is more pronounced in the presence of poly d(AT) than of poly d(GC) might indicate that DRB triphosphate is an analogue of ATP rather than of GTP.

However, the further investigations cast doubt on the simple explanation of the DRB effect outlined above. The results described in Fig. 2 and Fig. 3 clearly indicate that there is no preferential inhibition of the initiation reaction. They are more compatible with a reduction of the rate of elongation by DRB triphosphate.

The high concentration of DRB triphosphate required indicates a rather low affinity of the inhibitor for the polymerase. If inhibition were at the triphosphate level then one might expect that a measurable amount of DRB would be phosphorylated in vivo. However, DRB does not accumulate in the cells to a measurable extent and remains at an intracellular concentration far below that of ATP. We therefore conclude that the inhibition of RNA synthesis in vivo does not result from the effects of DRB triphosphate found in the in vitro systems. Consistent with this argument is the rapid reversibility of the in vivo effect of DRB (7); nucleoside triphosphates are normally not released from the cells.

The observation that both 5'pppG and 5'pppA ends are found labeled in the hn RNA resistant to DRB (Fig. 6) shows that normal RNA chain initiations with both purine nucleotides can occur in the presence of the drug. Thus the fact that DRB affects only a fraction of hn RNA synthesis cannot be explained by an interference with either A or G initiations.

Recent experiments on transcription of the adenovirus genome support our conclusion that the effect of DRB cannot be on the RNA chain initiation reaction. It was found that the point of DRB action is located a few hundred nucleotides downstream from the 5' end of the primary transcript (25, 26). Further investigations on the metabolism of the drug by using radioactive DRB may elucidate what is the active compound in vivo and what is the reaction inhibited by this compound. Experiments in this direction have so far been hampered by the instability of DRB during the attempt of catalytic exchange labeling with tritium.

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