MOLECULAR AND CELLULAR BIOLOGY, Apr. 1982, p. 467–477
0270-7306/82/040467-11$02.00/0

Vol. 2, No. 4

# Isolation and Characterization of 5,6-Dichloro-1-β-D-Ribofuranosylbenzimidazole-Resistant Mutants of the Chinese Hamster Ovary Cell Line

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Received 2 June 1981/Accepted 7 January 1982

Mutants resistant to the RNA synthesis inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) have been isolated in the Chinese hamster ovary cell line CHO-K1. Three independently isolated mutants, DRB6, DRB10, and DRB13, were 3-, 5-, and 3.5-fold, respectively, more resistant to DRB than the parental cell line WTCHO. The DRB-resistant mutations were expressed codominantly in somatic cell hybrids of DRB-resistant and DRB-sensitive cell lines. In vivo treatment of CHO-K1 cells with DRB resulted in specific inhibition of endogenous RNA polymerase II activity in cell lysates. Whereas DRB inhibited RNA polymerase II activity in WTCHO cells by a maximum of 60% at concentrations as low as 60 μM, 300 μM DRB was required to inhibit 60% of the RNA polymerase II activity in DRB10 cells. However, the inhibition of the DRB-sensitive RNA polymerase II activity in DRB10 was biphasic. About half (53 to 56%) of this activity was inhibited by 90 μM DRB and thus showed a DRB sensitivity similar to the wild-type RNA polymerase II activity; the remaining DRB-sensitive RNA polymerase II activity was maximally inhibited by 300 μM DRB. These results indicated that there were two copies of the *drb*$^R$ locus (*drb*$^+$ and *drb*$^R$*-10*) in DRB10 and confirmed that the *drb*$^R$*-10* mutation was expressed codominantly. Somatic cell hybrids of DRB-resistant and α-amanitin-resistant cell lines grew in medium containing both DRB and α-amanitin, demonstrating that the *drb*$^R$ and *ama*$^R$ mutations were not in the same gene. Thus, the *drb*$^R$ mutations may define an additional component of the RNA polymerase II transcriptional complex in mammalian cells.

The components that comprise a functional RNA polymerase II (nucleoside triphosphate: RNA nucleotidyl transferase, EC 2.7.7.6) transcriptional complex in eucaryotic cells are not well defined. Although purified RNA polymerase II will accurately initiate transcription at the promoters for the conalbumin, ovalbumin, and globin genes in soluble cell-free extracts from cultured mammalian cells (26, 40), at least four other components in the soluble extract are necessary for the selective initiation of transcription (27). In addition, purified RNA polymerase II is a structurally complex enzyme composed of at least 10 subunits, the functions of which are largely unknown (33).

In procaryotes, mutations affecting RNA polymerase have been used to characterize the subunits of the RNA polymerase holoenzyme and identify regulatory factors associated with the RNA polymerase transcriptional complex (reviewed in 41). Several of the mutations in RNA polymerase have been obtained by selecting for mutants resistant to inhibitors of RNA synthesis. However, in eucaryotic cells, there are three functionally and structurally distinct RNA polymerases (4, 33). RNA polymerase I is located in the nucleolus and synthesizes rRNA; RNA polymerases II and III are found in the nucleoplasm and synthesize heterogeneous nuclear RNA (hnRNA) and low-molecular-weight RNAs, respectively. Therefore, specific inhibitors of hnRNA synthesis are needed to select for mutations affecting RNA polymerase II.

The mushroom poison α-amanitin inhibits hnRNA chain elongation by irreversibly binding to the 140,000-dalton subunit of RNA polymerase II (2). Mammalian cell mutants resistant to α-amanitin have altered RNA polymerase II enzymes, which do not bind α-amanitin as effectively as the wild-type enzyme does (5, 23). Since α-amanitin inhibits elongation of hnRNA chains by RNA polymerase II, α-amanitin-resistant (Ama$^R$) mutations may identify the subunit of RNA polymerase II involved in elongation. Additional inhibitors of hnRNA synthesis would

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be useful for defining other components of RNA polymerase II and for assigning functional roles to those components.

An inhibitor of such potential is the adenosine analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which selectively inhibits 60 to 75% of hnRNA synthesis in mammalian cells (34, 37). DRB appears to inhibit hnRNA synthesis by enhancing premature termination of transcription near promoter-proximal regions of structural genes (38, 39). It is not clear whether DRB directly interacts with the RNA polymerase II transcriptional complex to terminate transcription, because DRB does not inhibit hnRNA synthesis in vitro (9, 36). Unlike adenosine and some of its analogs, DRB does not have to be phosphorylated to exert its inhibitory effect (10, 19), nor does it alter the ribonucleotide precursor pools (20). Thus, the molecular basis for termination of transcription by DRB is not known.

Mutants of somatic cells have been used to define the mechanism of action of inhibitors of RNA (5, 17) and protein (16, 18, 28) synthesis. Mutants of mammalian cells resistant to DRB would be useful for determining the biochemical action of DRB. Gupta and Siminovitch (19) recently isolated DRB-resistant ($Drb^R$) mutants of the Chinese hamster ovary (CHO) cell line and attempted to characterize the biochemical alteration in one of the $Drb^R$ mutants ($Drb^R8$). These investigators showed that total RNA synthesis was more resistant to inhibition by DRB in $Drb^R8$ than in the wild-type cell line. However, the inhibition of total RNA synthesis in DRB-treated cells could not be used to approximate the resistance of RNA polymerase II activity to inhibition by DRB, since DRB also directly or indirectly (or both) inhibits rRNA synthesis (12, 20). In addition, DRB did not inhibit the in vitro activity of RNA polymerase II from either the wild-type or $Drb^R$ cells. Therefore, another approach was needed to characterize the alterations in the $Drb^R$ mutants.

In this paper, I report the use of an in vitro transcription system, described by Dreyer and Hausen (9), to specifically assay RNA polymerase II activity in DRB-treated cells. RNA polymerase II activity was assayed in cell lysates by determining the amount of in vitro RNA synthesis that was sensitive to 1 μg of α-amanitin per ml (33). Dreyer and Hausen (9) have shown that pretreatment of Ehrlich ascites cells with DRB in vivo causes specific inhibition of endogenous RNA polymerase II activity in cell lysates. The results in this study show that RNA polymerase II activity in wild-type CHO (WTCHO) cells is also specifically inhibited by DRB. Furthermore, the dose-response effect of DRB on RNA polymerase II activity in WTCHO cells was similar to the dose-response effect of DRB on hnRNA synthesis, which has been reported for several other mammalian cell lines (37). Thus, the determination of RNA polymerase II activity in lysates of DRB-treated cells provided a valid means for assaying the effect of DRB on hnRNA synthesis and was used in the present study to characterize the resistance of RNA polymerase II activity to inhibition by DRB in a $Drb^R$ mutant (DRB10), whose isolation and characterization are also reported in this study.

(Part of this work has been presented previously [V. L. Funanage, 1980, Genetics **94**:S34–S35] and as a dissertation [1981] submitted to the University of Delaware in partial fulfillment of the requirements for the Ph.D. degree.)

## MATERIALS AND METHODS

**Materials.** DRB was obtained from Calbiochem-Behring Corp. (La Jolla, Calif.). Stock solutions of DRB were dissolved in 4 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) containing 50% (vol/vol) dimethyl sulfoxide. Ethyl methane sulfonate (EMS), α-amanitin, actinomycin D, creatine phosphokinase (160 U/mg), and saponin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Polyethylene glycol (molecular weight, 1,000) was from J. T. Baker Chemical Co. (Phillipsburg, N.J.), Tween-80 came from Fisher Scientific Co. (Fair Lawn, N.J.), and [5,6-$^3$H]uridine-5′-triphosphate (36 Ci/mmol) was purchased from ICN Chemical and Radioisotope Division (Irvine, Calif.). Other chemicals were of the highest grade commercially available.

**Cell lines.** The wild-type cell line (WTCHO) used in this study is a clonal isolate ($BH_4$) of CHO-K1 (22) and was provided by J. Irr, Haskell Laboratory, E. I. du Pont de Nemours & Co., Inc. (Wilmington, Del.). $AdeA^-$ and $AdeC^-$ are adenine auxotrophs of the CHO-K1 cell line and were supplied by D. Patterson, University of Colorado Medical Center (Denver, Colo.).

**Media and growth of cultures.** Cells were routinely grown in Ham F12 medium supplemented with 10% (vol/vol) fetal calf serum (F12FCS10). In several experiments, 10% dialyzed fetal calf serum was substituted for 10% fetal calf serum, and this medium was designated as F12dFCS10. Cells were maintained as monolayer cultures at 37°C in a humidified atmosphere of 5% $CO_2$ in air. For the RNA polymerase assays, cells were grown in suspension culture at 37°C in F12 medium supplemented with 5% (vol/vol) fetal calf serum.

Cells were examined for mycoplasma contamination by in situ staining with the DNA-binding dye Hoechst 33258, as previously described (6). The wild-type and $Drb^R$ cells were not infected with mycoplasma.

**Dose-response curves.** Cells (200 to 1,000) were plated with various concentrations of DRB or solvent control (4 mM HEPES with 50% dimethyl sulfoxide) in 60-mm tissue culture dishes containing 5 ml of F12FCS10 medium. After 7 to 10 days, the colonies were fixed for 20 min in 2 ml of absolute methanol and stained for 1 h in 5 ml of 5% (wt/vol) Giemsa stain. A colony was defined as a cluster of at least 50 cells. The

plating efficiency was determined by calculating the ratio of the number of colonies to the number of cells plated. For determination of relative plating efficiency, the plating efficiency of the drug-treated cells was expressed relative to the plating efficiency of untreated control cells.

**Mutagenesis and selection.** Cells were mutagenized with EMS (42 to 667 μg/ml) as described by Hsie et al. (22). One million cells from each independently mutagenized population were subcultured for a 7-day expression time. The mutagenized cells were then plated at a density of $2 \times 10^5$ cells per 100-mm tissue culture dish in 10 ml of either F12dFCS10 medium containing 105 μM DRB for the selection of $Drb^R$ mutants or F12FCS10 medium containing 1 μg of α-amanitin per ml for the isolation of $Ama^R$ mutants.

**Somatic cell hybridization.** The protocol for hybridization was a modification of that described by Davidson et al. (8). Cells ($5 \times 10^5$) from both parental cell lines were plated together in 25-$cm^2$ flasks containing 5 ml of F12FCS10 medium. After 24 h, the monolayers were washed once with 2 ml of Dulbecco minimal essential medium, and 3 ml of 50% (wt/wt) polyethylene glycol, dissolved in Dulbecco minimal essential medium containing 15% (vol/vol) dimethyl sulfoxide, was added to each flask. After 1 min at room temperature, the polyethylene glycol was removed, and the monolayers were rapidly rinsed 5× with Puck saline G. F12FCS10 medium (5 ml) was added to each flask, and the cells were incubated at 37°C for 24 h. The cells were then harvested by trypsinization and seeded at densities of $5 \times 10^3$, $1 \times 10^4$, and $5 \times 10^4$ per 100-mm tissue culture dish; each dish contained 10 ml of selective medium, which is described for each cross (e.g., AMA2 × $AdeC^-$) in the text. Colonies were picked after 7 to 10 days and tested for the expression of other genetic markers as described in the Results.

**Pretreatment of cells with DRB.** Cells (~$5 \times 10^5$/ml) were harvested by centrifugation and suspended at $4 \times 10^6$ cells per ml in fresh F12FCS10 medium. Ten-milliliter samples were then distributed to tubes fitted with magnetic stirring bars and containing 0.1 ml of the appropriate concentration of either DRB or 4 mM HEPES–50% dimethyl sulfoxide. Unless stated otherwise, cells were treated with DRB for 30 min at 37°C. The cells were then cooled to 0°C, washed twice with ice-cold saline G, and resuspended at $1 \times 10^8$ to $2 \times 10^8$ cells per ml in buffer A (10 mM Tris-hydrochloride [pH 7.8]–20 mM KCl–240 mM sucrose–2 mM $MgCl_2$–0.1 mM dithiothreitol–5 mM mercaptoethanol) as described by Dreyer and Hausen (9).

**RNA polymerase assay.** RNA polymerase activity was assayed as described (9), except that mercaptoglycerol was replaced with mercaptoethanol, and the ammonium sulfate concentration was 50 instead of 25 mM, unless stated otherwise. Assays were performed in triplicate in 100-μl volumes. The reaction mixture contained 50 mM Tris-hydrochloride (pH 7.8), 240 mM sucrose, 500 μM each of ATP, UTP, CTP, and GTP, 2 mM $MnCl_2$, 100 μM dithiothreitol, 5 mM mercaptoethanol, 20 mM creatine phosphate, 500 μg of creatine kinase per ml, 20 μCi of [$^3$H]UTP per ml, and $4 \times 10^6$ to $8 \times 10^6$ cells. α-Amanitin (1 μg/ml) was included in the reaction mixture to specifically inhibit RNA polymerase II activity. Cells (40 μl) were preincubated in 50 μl of reaction mixture for 1 min at 25°C. The reaction was initiated with 10 μl of 10% (wt/vol) saponin and terminated after 10 min with 1 ml of 10% (wt/wt) trichloroacetic acid containing 30 mM tetrapotassium pyrophosphate. After 30 min on ice, the precipitates were collected on 25-mm Whatman glass fiber filters and washed with 30 ml of ice-cold 5% (wt/wt) trichloroacetic acid. The filters were dried under an infrared lamp and placed in scintillation vials containing 0.5 ml of Protosol (New England Nuclear Corp., Boston, Mass.). After 30 min at 60°C, the Protosol was neutralized with 100 μl of glacial acetic acid, and the solubilized precipitates were counted in 10 ml of Triton-Omnifluor scintillation fluid. RNA polymerase II activity was determined from the amount of acid-precipitable radioactivity that was sensitive to 1 μg of α-amanitin per ml.

## RESULTS

**Isolation of DRB-resistant mutants.** WTCHO cells were plated in various concentrations of DRB to determine the dose of DRB needed to kill the wild-type cells (Fig. 1). About 1% of the WTCHO population survived in 60 μM DRB; however, the plating efficiency of the WTCHO cells was reduced to $<10^{-6}$ in 90 μM DRB.

Colonies spontaneously resistant to 105 μM DRB were observed at a frequency of $5 \times 10^{-7}$. If the resistance to DRB had a mutational basis, the number of $Drb^R$ colonies in independent

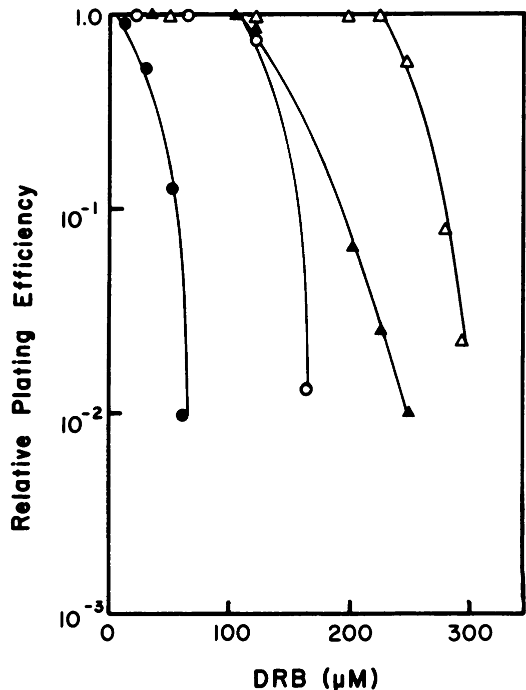


FIG. 1. Dose-response curves of wild-type and $Drb^R$ cells in DRB. Exponentially growing cells were plated in medium containing the indicated concentrations of DRB. After 7 days, the colonies were fixed, stained, and counted as described in the text. Symbols: ●, WTCHO; ○, DRB6; ▲, DRB13; △, DRB10.

cultures should fluctuate; however, if DRB resistance was acquired by WTCHO cells from exposure to DRB, the number of Drb$^R$ colonies in independent cultures should show a narrow distribution around the mean number of Drb$^R$ colonies. A Luria-Delbrück fluctuation analysis (25) showed that the number of Drb$^R$ colonies in 40 independent cultures varied significantly (Table 1), demonstrating that mutation was the basis for DRB resistance.

WTCHO cells were mutagenized with EMS to increase the frequency of DRB resistance, and Drb$^R$ mutants were selected from several independently mutagenized populations. The number of Drb$^R$ colonies showed a linear increase in response to increasing concentrations of EMS (data not shown), in agreement with previous results (19). Three Drb$^R$ mutants DRB6, DRB10, and DRB13 were isolated from independently mutagenized cultures. DRB6, DRB10, and DRB13 were 3-, 5- and 3.5-fold, respectively, more resistant to DRB than WTCHO (Fig. 1) was. After 6 months in continuous culture without DRB, the mutants maintained the same relative degree of resistance to DRB, indicating that the *drb*$^R$ mutations were stable.

**Cross-resistance of Drb$^R$ mutants to other inhibitors.** An alteration in drug permeability has been shown to account for the phenotype of several drug-resistant mutants of mammalian somatic cells (11, 21, 24, 32). Since a radiolabeled preparation of DRB was not commercially available, alternative methods were used to determine if the Drb$^R$ mutants were unable to transport DRB. DRB is an adenosine analog which competitively inhibits the transport of nucleosides into cells (37); thus, the mutants could be resistant to DRB because of a defect in nucleoside transport.

Cohen et al. (7) isolated a mouse lymphoma mutant cell line which does not transport nucleosides and is resistant to adenosine and 5-fluoro-2′-deoxyuridine. The Drb$^R$ mutants were not resistant to either adenosine or 5-fluoro-2′-deoxyuridine, and the uptake of uridine was also similar in the wild-type and Drb$^R$ cells (data not shown). These results indicated that an inability to transport nucleosides did not account for the Drb$^R$ phenotype.

The resistance of the mutants to DRB was also tested under conditions whereby DRB would be expected to enter the mutant cells. The nonionic detergent Tween-80 increases the permeability of CHO cells to colchicine (24), actinomycin D (32), daunomycin (32), and nucleoside triphosphates (1). Also, it has been shown that 1 mg of Tween-80 per ml permeabilizes CHO cells to nucleoside triphosphates at 37°C, yet the cells maintain their ability to grow when plated at a density of at least $10^4$ cells per cm$^2$ (V. L. Funanage, Ph.D. dissertation, University of Delaware, Newark, 1981). This provided a means for determining whether the Drb$^R$ phenotype was due to an inability of the mutant cells to transport DRB.

In the presence of 1 mg of Tween-80 per ml, the morphology of the wild-type and mutant cells changed; the cells became elongated and filled with vacuoles (Fig. 2a,c). When the wild-type cells were exposed to 90 μM DRB in the presence of 1 mg of Tween-80 per ml, the cells died after a 24-h exposure to DRB. During this time, the nucleolus fragmented and was absent in most cells (Fig. 2b), demonstrating that the cytotoxicity of DRB was due to an inhibition of RNA synthesis in permeabilized cells (see 13). In contrast, the Drb$^R$ mutants were resistant to 90 μM DRB when permeabilized by Tween-80 (Fig. 2d). This indicated that the mutants were not resistant to DRB because of an inability of DRB to enter the mutant cells.

**Genetic analysis of DRB resistance.** Complementation testing of *drb*$^R$ and *ama*$^R$ mutations was used to determine whether the *drb*$^R$ mutations altered the α-amanitin-binding subunit of RNA polymerase II. For this analysis, it was necessary to isolate Drb$^R$ and Ama$^R$ mutants from cell lines with genetic markers useful for

TABLE 1. Fluctuation analysis of DRB resistance

| Culture[a] | No. of replicate cultures | No. of replicates with Drb$^R$ colonies | | | | | | | Mean | Variance | Variance/ mean | Mutation rate[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0[c] | 1[c] | 2[c] | 4[c] | 5[c] | 6[c] | 12[c] | | | | |
| Experimental | 40 | 22 | 6 | 7 | 1 | 2 | 1 | 1 | 1.3 | 5.5 | 4.2 | $1.0 \times 10^{-6}$ |
| Control | 20 | 18 | 2 | 0 | 0 | 0 | 0 | 0 | 0.1 | 0.1 | 1.0 | |

[a] Forty 35-mm tissue culture dishes were each seeded with 200 WTCHO cells. The cultures were grown to a density of $\sim 6 \times 10^5$ cells per dish, and $4 \times 10^5$ cells from each sample were inoculated into 100-mm tissue culture dishes containing 10 ml of F12dFCS10 medium and 105 μM DRB. A control culture was grown from 200 to $5 \times 10^6$ cells, and $2 \times 10^5$ cells were distributed into each of 20 100-mm tissue culture dishes containing the same medium. The number of Drb$^R$ colonies was scored after 10 days.

[b] The mutation rate is expressed as mutations per cell per generation and was determined from the fraction of cultures with no Drb$^R$ colonies as previously described (35).

[c] Number of Drb$^R$ colonies.

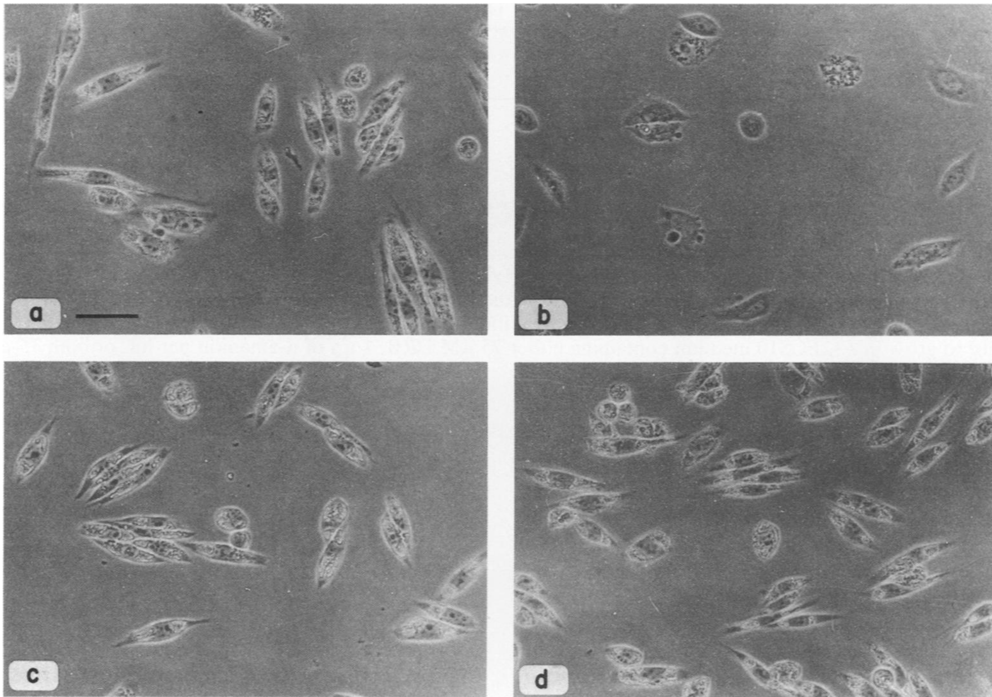


FIG. 2. Phase-contrast photomicrographs of wild-type and mutant cells permeabilized in the presence and absence of DRB. Cells ($2.5 \times 10^5$) were plated in 60-mm tissue culture dishes containing 5 ml of F12FCS10 medium and 1 mg of Tween-80 per ml, with or without 90 μM DRB. For this experiment, DRB was dissolved directly in the medium, since HEPES was toxic to cells in the presence of Tween-80 (unpublished observation). After 24 h at 37°C, phase-contrast photomicrographs were taken by removing most of the medium from each dish; a thin layer of medium was left to cover the cells. An open dish was then placed under the phase-contrast objective (×16) of a Zeiss photomicroscope II, and cells were photographed with Panatomic X film. (a) WTCHO, −DRB; (b) WTCHO, +DRB; (c) DRB6, −DRB; (d) DRB6, +DRB. Bar represents 50 μm.

the selection of somatic cell hybrids. AdeA$^-$ and AdeC$^-$ are adenine auxotrophs (Ade$^-$) of the CHO-K1 cell line which lack phosphoribosylpyrophosphate amidotransferase and glycinamide ribonucleotide synthetase activities, respectively (29). Somatic cell hybrids of AdeA$^-$ and AdeC$^-$ grow in medium lacking purines, indicating that the *ade* mutations complement (30).

AdeA$^-$ and AdeC$^-$ were mutagenized with 200, 400, and 600 μg of EMS per ml. The survival of AdeC$^-$ after EMS mutagenesis was low, and it was not possible to obtain mutants (data not shown). However, Drb$^R$ and Ama$^R$ mutants were obtained from AdeA$^-$ at a frequency of $4.3 \times 10^{-5}$ and $3.1 \times 10^{-5}$, respectively, after mutagenesis by 200 μg of EMS per ml. One Drb$^R$ mutant, DRB103, and one Ama$^R$ mutant, AMA2, were chosen for further studies.

The expression of the mutations in DRB103 and AMA2 was determined by hybridizing each drug-resistant mutant with AdeC$^-$. Since the G-banded karyotypes of DRB103, AMA2, and AdeA$^-$ differed from the G-banded karyotype of AdeC$^-$ (Funanage, Ph.D. dissertation), the marker chromosomes in AdeC$^-$ and in the mutant cell lines derived from AdeA$^-$ were used for the identification of hybrids. Cross A in Table 2 shows that AdeA$^-$ × AdeC$^-$ hybrids were not resistant to either DRB or α-amanitin. Cross B (DRB103 × AdeC$^-$) resulted in hybrids resistant to DRB, indicating that the *drb*$^R$-*103* mutation was dominant. In cross C (AMA2 × AdeC$^-$), the hybrids were resistant to α-amanitin; thus, the *ama*$^R$-*2* mutation was also expressed dominantly.

The dominance of the *ama*$^R$-*2* mutation, coupled with the recessive expression of the *adeA10* mutation in AMA2, allowed a genetic analysis of the *drb*$^R$ mutations in DRB6 and DRB10. In medium lacking purines and containing 1 μg of α-amanitin per ml, neither AMA2 nor the Drb$^R$ mutants would grow. However, in the hybrids constructed between AMA2 and the Drb$^R$ mutants, AMA2 would contribute the ability to grow in α-amanitin, and the Drb$^R$ mutants would

TABLE 2. Genetic analysis of DRB and α-amanitin resistance[a]

| Cross | No. of hybrids analyzed | No. of hybrids that grew in: | | |
|---|---|---|---|---|
| | | DRB | α-AM | DRB + α-AM |
| A. AdeA$^-$ (*adeA10*) × AdeC$^-$ (*adeC10*) | 14 | 0 | 0 | 0 |
| B. DRB103 (*drb*$^R$*-103 adeA10*) × AdeC$^-$ (*adeC10*) | 6 | 6 | 0 | 0 |
| C. AMA2 (*ama*$^R$*-2 adeA10*) × AdeC$^-$ (*adeC10*) | 8 | 0 | 8 | 0 |
| D. WTCHO (*ade*$^+$) × AMA2 (*adeA10 ama*$^R$*-2*) | 14 | 0 | 14 | 0 |
| E. DRB6 (*ade*$^+$ *drb*$^R$*-6*) × AMA2 (*adeA10 ama*$^R$*-2*) | 11 | 11 | 11 | 11 |
| F. DRB10 (*ade*$^+$ *drb*$^R$*-10*) × AMA2 (*adeA10 ama*$^R$*-2*) | 11 | 11 | 11 | 11 |

[a] Cells were hybridized as described in the text. Hybrids from crosses A through C were selected for in F12dFCS10 medium without hypoxanthine. Hybrids from crosses D through F were selected for in F12dFCS10 medium without hypoxanthine and with 1 μg of α-amanitin (α-AM) per ml. The hybrids were then tested for their ability to grow in F12FCS10 medium containing either 75 μM DRB, 1 μg of α-amanitin per ml, or both 75 μM DRB and 1 μg of α-amanitin per ml.

contribute the necessary enzyme to grow in medium without purines.

The existence of hybrids was again confirmed for each cross (Table 2, crosses D, E, and F) by cytogenetic analysis, since the karyotypes of WTCHO, DRB6, and DRB10 differed from the karyotype of AMA2 (Funanage, Ph.D. dissertation). The control cross (Table 2, cross D) shows that although the WTCHO × AMA2 hybrids were resistant to α-amanitin, they were not resistant to DRB. However, the DRB6 × AMA2 (cross E) and DRB10 × AMA2 (cross F) hybrids were resistant to DRB, indicating that the *drb*$^R$*-6* and *drb*$^R$*-10* mutations were dominant. The resistance of the hybrids to DRB was more critically evaluated by determining dose-response curves (Fig. 3). The hybrids displayed a sensitivity to DRB intermediate between that of the parental cell lines, demonstrating that the *drb*$^R$ mutations were expressed in a codominant manner.

If the *drb*$^R$ and *ama*$^R$ mutations altered the same subunit of RNA polymerase II, the Drb$^R$ × Ama$^R$ hybrids would not grow in the presence of both DRB and α-amanitin. However, if the *drb*$^R$ and *ama*$^R$ mutations affected different subunits of RNA polymerase II, the DRB-resistant and the α-amanitin-resistant subunits might associate in the hybrids to yield some RNA polymerase II enzymes which are resistant to both DRB and α-amanitin. In this case, the Drb$^R$ × Ama$^R$ hybrids would grow in the presence of both DRB and α-amanitin. Similarly, if the *drb*$^R$ mutations altered a component other than RNA polymerase II, the Drb$^R$ × Ama$^R$ hybrids would grow in the presence of both drugs. The DRB6 × AMA2 and DRB10 × AMA2 hybrids grew in the presence of both DRB and α-amanitin (Table 2, crosses E and F), indicating that the *drb*$^R$ and *ama*$^R$ mutations were not allelic.

**Characterization of RNA polymerase activity in cell lysates.** Dreyer and Hausen (9) observed two different RNA polymerase activities in lysates of Ehrlich ascites cells. These activities differed in their salt optima (25 or 300 mM ammonium sulfate) and in their sensitivity to inhibition by DRB. Whereas the RNA polymerase activity at 25 mM was sensitive to DRB, the activity at 300 mM was highly resistant. To determine the effect of DRB on RNA polymerase II activity in DRB10 cells, it was necessary to first examine the effect of salt concentration on the RNA polymerase activity in lysates of WTCHO cells. Figure 4 shows that, in contrast to Ehrlich ascites cell lysates, extracts of WTCHO cells contained RNA polymerase activity with a single salt optimum of 25 mM ammonium sulfate.

The RNA polymerase activity at 25 mM ammonium sulfate consisted of RNA polymerase I, II, and III activities, which have been shown to represent 40, 50, and 10%, respectively, of the RNA polymerase activity in lysates from exponentially growing cells (9). To determine the salt optimum for RNA polymerase II activity, WTCHO cells were treated with 0.04 μg of actinomycin D per ml to inhibit rRNA synthesis by RNA polymerase I (31). Since there was only one salt optimum (25 mM ammonium sulfate) for

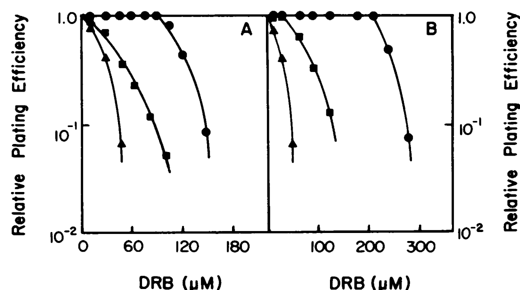


FIG. 3. Dose-response curves of hybrids of Drb$^R$ and Drb$^S$ cell lines in DRB. Exponentially growing cells were plated in the indicated concentrations of DRB. After 10 days, colonies were fixed, stained, and counted as described in the text. (A) Symbols: ▲, AMA2; ●, DRB6; ■, AMA2 × DRB6. (B) Symbols: ▲, AMA2; ●, DRB10; ■, AMA2 × DRB10.

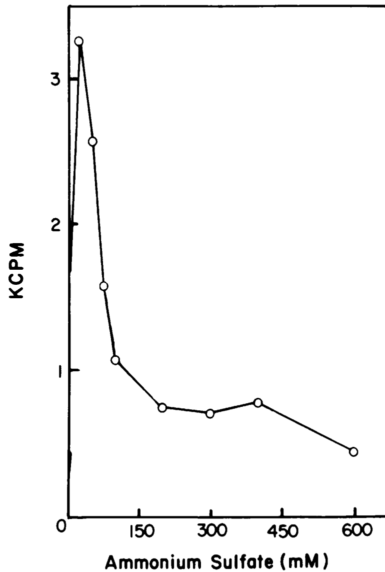


FIG. 4. Effect of ammonium sulfate on RNA polymerase activity in WTCHO cell lysates. RNA polymerase activity was assayed in the presence of the indicated ammonium sulfate concentrations as described in the text.

the total RNA polymerase activity in WTCHO cell lysates, the RNA polymerase activity in lysates of actinomycin D-treated cells was only assayed in the low salt range (0 to 75 mM ammonium sulfate). This activity showed a salt optimum at 50 mM ammonium sulfate (Fig. 5). A salt optimum at 50 mM ammonium sulfate was also observed for the RNA polymerase activities in lysates of actinomycin D-treated cells of DRB6 and DRB10. About 80% of the RNA polymerase activity in lysates of the actinomycin D-treated wild-type and $Drb^R$ cells was sensitive to 1 μg of α-amanitin per ml and therefore represented RNA polymerase II activity (data not shown).

**Effect of pretreatment of cells with DRB on RNA polymerase activities in cell lysates.** Since pretreatment of Ehrlich ascites cells with DRB in vivo completely inhibited RNA polymerase II activity at 25 mM ammonium sulfate (9), the effect of DRB on RNA polymerase II activity in WTCHO cells was determined. In contrast to the complete inhibition of RNA polymerase II activity by DRB at 25 mM ammonium sulfate in Ehrlich ascites cell lysates, RNA polymerase II activity in WTCHO cell lysates was only partially inhibited by pretreatment with 75 μM DRB for 15 min (Table 3). The RNA polymerase II activity was inhibited by about 60 and 70% when assayed at 25 and 50 mM ammonium sulfate, respectively. Pretreatment of WTCHO cells with DRB for a longer time (30 min) or with a higher concentration of DRB (90 μM) did not result in an increased inhibition of RNA polymerase II activity (data not shown). This result agrees with the observation that not all hnRNA synthesis (RNA polymerase II activity) is inhibited by DRB in mammalian cells (34, 37). Table 3 also shows that the α-amanitin-resistant RNA polymerase activity (RNA polymerases I and III) was not inhibited by a 15-min exposure to 75 μM DRB. However, after a 30-min exposure to 75 μM DRB, the RNA polymerase I and III activity in WTCHO cell lysates was inhibited by about 20% (data not shown).

**Dose-response effect of DRB pretreatment on RNA polymerase II activity in cell lysates of WTCHO and DRB10.** The inhibition of hnRNA synthesis by DRB in HeLa and mouse L cells has been shown to be dose dependent in the range of 5 to 30 μM DRB and maximally inhibited by 60 to 75% at 60 μM DRB (37). To characterize the dose-response effect of DRB on RNA polymerase II activity, WTCHO cells were pretreated with various concentrations of DRB, and RNA polymerase II activity was assayed in lysates of these cells (Fig. 6). Since

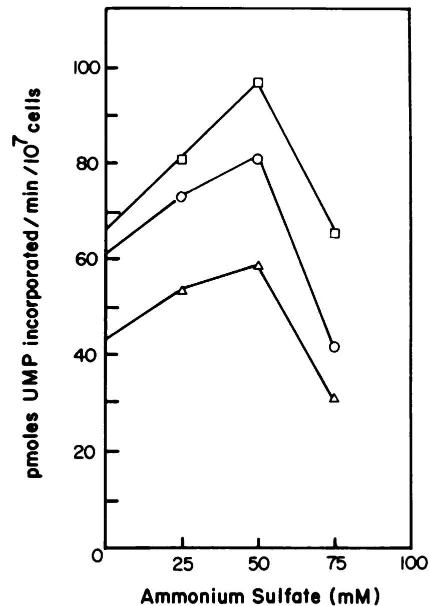


FIG. 5. RNA polymerase activities in lysates of wild-type and mutant cells pretreated with actinomycin D. Actinomycin D (0.04 μg/ml) was added directly to 1-liter suspension cultures of cells. After 30 min, the cells were harvested, washed, and assayed for RNA polymerase activity as described in the text except that 0.04 μg of actinomycin D per ml was also included in the reaction mixture. Symbols: ○, WTCHO; △, DRB6; □, DRB10.

TABLE 3. Effect of 75 μM DRB on RNA polymerase activities in WTCHO

| DRB (μM) | RNA polymerase II[a] | | RNA polymerase I and III[a] | |
|---|---|---|---|---|
| | 25 mM | 50 mM | 25 mM | 50 mM |
| 0 | 39 | 47 | 51 | 30 |
| 75 | 16 | 14 | 53 | 32 |

[a] Values represent picomoles of UMP incorporated per minute per $10^7$ cells at the indicated ammonium sulfate concentrations. RNA polymerase II activity was determined from the amount of acid-precipitable radioactivity that was sensitive to 1 μg of α-amanitin per ml. The α-amanitin-resistant acid-precipitable radioactivity represented the activities of RNA polymerase I and III.

WTCHO cells were killed by 90 μM DRB (Fig. 1), RNA polymerase II activity was not assayed at DRB concentrations above 90 μM. The RNA polymerase II activity was inhibited by about 35 and 55% at 12 and 30 μM DRB, respectively, and was maximally inhibited by 61% at 60 μM DRB. The RNA polymerase II activity that was resistant to DRB most likely represented the 25 to 40% of hnRNA which continues to be synthesized at DRB concentrations as high as 300 μM (34). Thus, the dose-response effect of DRB on RNA polymerase II activity was similar to the dose-response effect of DRB on hnRNA synthesis reported by others (37).

Less than $10^{-6}$ WTCHO cells survived in 90 μM DRB; in contrast, 230 μM DRB was needed to decrease the survival of DRB10 cells (Fig. 1). To determine whether the Drb$^R$ phenotype of DRB10 was due to a decrease in the sensitivity of RNA polymerase II activity to inhibition by DRB, RNA polymerase II activity was assayed in lysates from DRB10 cells which had been exposed to DRB concentrations ranging from 45 to 360 μM. In contrast to the monophasic inhibition of RNA polymerase II activity by DRB in WTCHO, the inhibition of RNA polymerase II activity in DRB10 was biphasic (Fig. 6A). The biphasic inhibition of RNA polymerase II activity by DRB suggested that two RNA polymerase II transcriptional complexes, differing in their sensitivity to DRB, were present in DRB10.

The DRB-resistant fraction (~41% of control) in DRB10 cells was similar to that observed in WTCHO cells. The biphasic inhibition of RNA polymerase II activity was therefore more clearly observed when the DRB-resistant RNA polymerase II activity was subtracted from each datum point in Fig. 6A and the values were replotted (Fig. 6B). About half (53 to 56%) of the DRB-sensitive RNA polymerase II activity (transcriptional complex-1) in DRB10 showed a wild-type sensitivity to DRB. The RNA polymerase II activity in WTCHO and transcriptional complex-1 in DRB10 were maximally inhibited at 60 and 90 μm DRB, respectively. The remaining DRB-sensitive RNA polymerase II activity (transcriptional complex-2) in DRB10 was maximally inhibited at 300 μM DRB. At this concentration of DRB, <$10^{-4}$ DRB10 cells survived (Fig. 1). Thus, the decrease in the activity of transcriptional complex-2 was directly correlated with the decrease in survival of DRB10 cells in DRB.

**Dose-response effect of DRB pretreatment on RNA polymerase I and III activities in cell lysates of WTCHO and DRB10.** Since DRB also inhibits rRNA synthesis (12, 20), it was important to determine the effect of DRB on the RNA polymerase I and III activities in WTCHO and DRB10 cells. After WTCHO cells were exposed to DRB concentrations ranging from 12 to 90 μM for 30 min, the RNA polymerase I and III activity was inhibited by only 20% (data not shown). Similarly, RNA polymerase I and III activity was inhibited by about 20% after DRB10 cells were exposed to DRB concentrations ranging from 45 to 360 μM for 30 min (data not shown). Since the inhibition of the RNA polymerase I and III activities in WTCHO and DRB10 cells was slight and not dose dependent, the survival of WTCHO and DRB10 cells in DRB was related to the inhibition of RNA polymerase II (hnRNA synthesis), not RNA polymerase I, activity (rRNA synthesis).

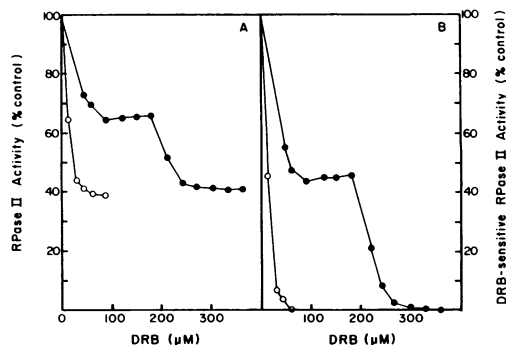


FIG. 6. Dose-response effect of DRB on RNA polymerase II activity in WTCHO and DRB10 cells. Cells were treated with the indicated concentrations of DRB, and RNA polymerase II activity was assayed as described in the text. Values in (A) are plotted as percent RNA polymerase II (RPase II) activity in control cells not exposed to DRB. Control values were 38 pmol of UMP incorporated per min per $10^7$ WTCHO cells and 58 pmol of UMP incorporated per min per $10^7$ DRB10 cells. The DRB-resistant RNA polymerase II activity (39% of control for WTCHO; 41% of control for DRB10) was subtracted from each datum point in (A); the resulting values are plotted in (B). Symbols: ○, WTCHO; ●, DRB10.

## DISCUSSION

Three Drb$^R$ mutants, varying in their relative resistance to DRB, have been isolated in the CHO-K1 cell line. Several observations indicated that mutation was the basis for the Drb$^R$ phenotype: (i) the number of Drb$^R$ colonies showed a linear increase in response to increasing concentrations of EMS; (ii) fluctuation analysis indicated that the spontaneous occurrence of DRB resistance was random and not induced by DRB; and (iii) the three Drb$^R$ mutants characterized here showed stable resistance to DRB after 6 months in continuous culture without DRB.

Resistance to DRB did not occur as a result of an alteration in nucleoside transport because the uptake of uridine was similar in WTCHO and the Drb$^R$ mutants, and the mutants were not cross-resistant to either adenosine or 5-fluoro-2′-deoxyuridine. The codominant expression of the *drb*$^R$ mutations provided further evidence for the mutations not affecting the transport of DRB, since most permease mutations in mammalian cells are recessive (7, 11, 21). Furthermore, the Drb$^R$ mutants were resistant to DRB when permeabilized to nucleoside triphosphates by Tween-80, which also increases the permeability of CHO cells to colchicine (24), actinomycin D (32), and daunomycin (32). Thus, if DRB must enter cells to inhibit hnRNA synthesis, these results suggest that the alterations in the Drb$^R$ mutants are in a step after DRB enters cells.

A loss of adenosine kinase activity has been shown to result in resistance to adenosine (17). Since the Drb$^R$ mutants were not cross-resistant to adenosine, a defect in adenosine kinase activity would not account for the Drb$^R$ phenotype of the mutants. This agrees with previous results (19) showing that mutants lacking adenosine kinase, hypoxanthine-guanine phosphoribotransferase, or adenine phosphoribotransferase activities are not resistant to DRB, suggesting that the toxicity of DRB is not due to phosphorylation of DRB by any of these enzymes. Furthermore, the AdeA$^-$ cell line used in this study needs hypoxanthine-guanine phosphoribotransferase activity to grow in F12 medium, and the isolation of DRB103, which still requires hypoxanthine for growth, indirectly suggests that a loss of hypoxanthine-guanine phosphoribotransferase activity does not account for the Drb$^R$ phenotype.

Somatic cell hybrids of DRB$^R$ and DRB-sensitive (DRB$^S$) cells showed an intermediate resistance to DRB (Fig. 3). Hybrids of Ama$^R$ and α-amanitin-sensitive (Ama$^S$) cells also show an intermediate resistance to α-amanitin, since they express both mutant and wild-type RNA polymerase II activities (23). Thus, it is reasonable to assume that both mutant and wild-type functions are expressed in the hybrids of DRB$^R$ and DRB$^S$ cells. This suggests that the Drb$^R$ mutants are not defective in an enzymatic activity necessary for converting DRB to an active form in vivo.

An in vitro transcription system described by Dreyer and Hausen (9) was used in this study to specifically assay RNA polymerase II activity in DRB-treated cells. Thus, it was possible to determine the in vivo effect of DRB on the RNA polymerase II activity in a Drb$^R$ cell line, DRB10. The RNA polymerase II activity in DRB10 showed a biphasic inhibition by DRB (Fig. 6). About half (53 to 56%) of the DRB-sensitive RNA polymerase II activity showed a wild-type sensitivity to DRB; the remaining DRB-sensitive RNA polymerase II activity was inhibited only at high concentrations of DRB (200 to 300 μM). The presence of two RNA polymerase II activities, differing in their sensitivity to DRB, confirmed that the *drb*$^R$*-10* mutation was expressed codominantly.

Although half of the DRB-sensitive RNA polymerase II activity in DRB10 was inhibited by 90 μM DRB (Fig. 6B), the survival of DRB10 cells in 90 μM DRB was not affected (Fig. 1). Two observations may explain this result. First, in WTCHO cells, the DRB-sensitive RNA polymerase II activity was inhibited by about 45% in 12 μM DRB (Fig. 6B); however, the survival of WTCHO cells in 12 μM DRB was only reduced by 8% (Fig. 1). Thus, a partial loss of RNA polymerase II activity may not be detrimental to the survival of either WTCHO or DRB10 cells. Second, in the absence of DRB, RNA polymerase II activity was 1.5- to 2-fold higher in DRB10 than in WTCHO cells, even though RNA polymerase I and III activities were similar (Funanage, Ph.D. dissertation). Thus, the increase in RNA polymerase II activity may enable DRB10 cells to survive when approximately 50% of their RNA polymerase II activity is inhibited by DRB. It may also explain why 90 μM DRB was required to maximally inhibit the activity of transcriptional complex-1 in DRB10, whereas 60 μM DRB was sufficient to inhibit the RNA polymerase II activity in WTCHO.

The activity of transcriptional complex-2 in DRB10 was maximally inhibited by 300 μM DRB. At this concentration of DRB, $<10^{-4}$ DRB10 cells survived. Thus, the decrease in the activity of transcriptional complex-2 was directly correlated with the decrease in survival of DRB10 cells in DRB. A direct correlation between α-amanitin resistance and the resistance of RNA polymerase II activity to inhibition by α-amanitin has also been observed for Ama$^R$ mutants of CHO cells (23). The Ama$^R$ mutants show different degrees of resistance to α-amani-

tin, and the differences in α-amanitin resistance are reflected in differences in the abilities of the mutant RNA polymerases to bind α-amanitin. By analogy with the Ama$^R$ mutants, it is reasonable to suggest that in DRB10, a component of transcriptional complex-2 is altered in its ability to bind the inhibitor of hnRNA synthesis in DRB-treated cells.

The *ama*$^R$ locus has been shown to be present in only one functional copy in CHO-K1 cells (15). The RNA polymerase II activity in Ama$^R$ mutants is totally resistant to α-amanitin, and therefore, the inhibition of RNA polymerase II activity by α-amanitin is monophasic. However, in lysates from DRB10 cells, the inhibition of RNA polymerase II activity by DRB was biphasic (Fig. 6). A biphasic inhibition curve has been observed for hybrids of Ama$^R$ and Ama$^S$ cell lines (23) and for a 1:1 mixture of RNA polymerase II from Ama$^R$ and Ama$^S$ cells (15). A biphasic inhibition curve has also been reported for Ama$^R$ mutants of *Drosophila melanogaster* (14) and human fibroblasts (3), in which two copies of the *ama*$^R$ locus are present. Therefore, the biphasic inhibition of RNA polymerase II activity by DRB in DRB10 suggests that there are two copies of the *drb*$^R$ locus in the CHO-K1 cell line. The difference in the number of functional gene copies for the *ama*$^R$ and *drb*$^R$ loci indicates that *ama*$^R$ and *drb*$^R$ mutations define different genes.

This hypothesis was supported by the results (Table 2) which indicated that the *drb*$^R$*-6* and *drb*$^R$*-10* mutations complemented the *ama*$^R$*-2* mutation. The most likely basis for the Ama$^R$ phenotype of AMA2 is an alteration in the α-amanitin-binding subunit of RNA polymerase II, since the *ama*$^R$*-2* mutation was expressed dominantly (Table 2), and all *ama*$^R$ mutations characterized in CHO cells have been shown to be dominant and allelic, and to alter RNA polymerase II (23). Since α-amanitin binds to the 140,000-dalton subunit of RNA polymerase II (2) and there is only one 140,000-dalton subunit present in purified RNA polymerase II (33), the results of the complementation tests indicate that the *drb*$^R$*-6* and *drb*$^R$*-10* mutations are not in the gene coding for the α-amanitin-binding subunit of RNA polymerase II. Thus, the *drb*$^R$ mutations may define an additional component of the RNA polymerase II transcriptional complex.

The altered response of RNA polymerase II activity to inhibition by DRB in DRB10 cells is consistent with the proposal that a component of the RNA polymerase II transcriptional complex is altered to confer DRB resistance. However, it was not possible to prove that this was the case, since 90 μM DRB did not inhibit RNA polymerase II activity when added directly to lysates from either WTCHO or DRB10 cells (Funanage, Ph.D. dissertation). Thus, identification of the biochemical alteration in DRB10 must await the development of an in vitro transcription assay which is sensitive to inhibition by DRB or its metabolite.

Previous studies (20, 37) have demonstrated that DRB also inhibits rRNA synthesis in mammalian cells. However, it had not been determined whether the inhibition of rRNA synthesis was due to a direct or indirect effect of DRB. In this work, a correlation between the survival of WTCHO cells in DRB and the resistance of RNA polymerase II activity to inhibition by DRB was observed. In agreement with the results in Ehrlich ascites cells (9), the α-amanitin-resistant RNA polymerase activity (RNA polymerases I and III) was not inhibited by a 15-min exposure to DRB in CHO cells. After a 30-min exposure to DRB, there was only a slight effect on RNA polymerase I and III activity. These results, in conjunction with the study of Granick (12), suggest that DRB indirectly inhibits rRNA synthesis. Furthermore, the increased survival of DRB10 cells in DRB was correlated with a change in the response of RNA polymerase II activity to inhibition by DRB. This implies that a direct inhibition of hnRNA synthesis, not rRNA synthesis, causes the growth-inhibitory effect of DRB in mammalian cells.

## ACKNOWLEDGMENTS

I am greatly indebted to Arnold M. Clark, in whose laboratory this work was performed, for his sponsorship and support. Some of the mutagenesis experiments were performed while I was a visiting researcher at Haskell Laboratory, E. I. du Pont de Nemours and Co., Inc., and I thank J. Irr and the staff at Haskell Laboratory for their interest in this project. I am grateful to A. McCracken, P. Harlow, D. Simmons, and K. Tweeten for their critical reading of this manuscript and to M. Boyer for typing the manuscript.

This work was supported by Human Heredity and Cell Control grants from the University of Delaware and by Public Health Service biomedical support grant 5 S07 RR07016-06 from the Division of Research Resources, National Institutes of Health.

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