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28 April 1976

5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole Inhibits Initiation of Nuclear Heterogeneous RNA Chains in HeLa Cells

Abstract. *The nucleoside analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) at 75 to 150 micromolar concentrations inhibits the synthesis of nuclear heterogeneous RNA (hnRNA) in HeLa cells by 60 to 70 percent. The sedimentation profile of hnRNA labeled with [³H]uridine for 45 seconds after brief treatment (45, 90, or 180 seconds) with DRB showed a progressive decrease in the labeling of shorter hnRNA molecules relative to longer molecules. Prior exposure of the cells to actinomycin D, an inhibitor of RNA chain elongation, did not alter the sedimentation profile of hnRNA. These results suggest that DRB preferentially inhibits the initiation of hnRNA chains so that after exposure to DRB for a brief period the longer nascent chains still remain to be finished and thus incorporate a greater share of the pulse label. By progressively increasing the time of exposure to DRB, and measuring the rate of increase in the average size of the labeled, nascent RNA, it was estimated that the chains were growing at rates between 50 and 100 nucleotides per second.*

The halobenzimidazole riboside 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) is a selective and reversible inhibitor of nuclear heterogeneous RNA (hnRNA) synthesis in human (HeLa), murine (L), avian (chicken), and insect (chironomid) cells (*1*, *2*). We have attempted to determine the mechanism by which DRB inhibits the synthesis of hnRNA molecules in mammalian cells.

In salivary gland cells of *Chironomus tentans*, DRB appears to inhibit hnRNA synthesis by preventing the initiation of RNA chains without affecting the completion of nascent, or incomplete, chains (*3*). Very brief nucleoside ([³H]uridine) incorporation by HeLa cells (*4*) permits the analysis of nascent RNA molecules. We have used these short-term labeling techniques to determine the effects of DRB on the synthesis of hnRNA in HeLa cells, and have found that DRB selectively inhibits the initiation of at least some of the hnRNA chains.

HeLa S3 cells were grown in suspension culture as described (*2*, *5*). The procedures for labeling hnRNA with [³H]uridine (New England Nuclear, 28.3 c/mmole), and for cell fractionation, RNA extraction, denaturation with 80 percent dimethyl sulfoxide, and sedimentation analysis in sucrose gradients have also been described (*2*, *4*, *5*). Because DRB inhibits the transport of nucleosides (*2*, *6*), it was necessary to make a substantial correction for the effect of DRB on the transport of [³H]uridine (*2*, *6*). In order to evaluate the inhibitory effect of DRB on hnRNA synthesis we determined the effect of DRB on hnRNA synthesis under conditions where the compound would have no effect on the transport of labeled precursor.

Table 1, experiment A, illustrates the inhibition by DRB (150 μ*M*) of the synthesis of hnRNA after the cells were exposed to [³H]uridine for 15 minutes; experiment A also shows the inhibition of [³H]uridine uptake by DRB. Cells treated with a low concentration of actinomycin D (0.05 μg/ml; Merck Sharp & Dohme) under these conditions incorporate more than 90 percent of the ³H into hnRNA (*2*, *7*). Labeling of hnRNA was also assayed (Table 1, experiment B) by [³H]uridine incorporation into DRB-treated and -untreated control cells in the presence of 300 μ*M* uridine. In experiment C, cells were exposed to [³H]-uridine at 4°C for 1 hour and then incubated for 15 minutes at 37°C in the presence or absence of DRB. Both these procedures effectively eliminated the inhibition of nucleoside transport but still demonstrated that 150 μ*M* DRB inhibits close to two-thirds of the labeling of hnRNA. The inhibitory effect of 75 μ*M* DRB on hnRNA synthesis was similar to that of 150 μ*M* DRB (data not shown). A similar inhibition of hnRNA synthesis also occurred when [³²P]phosphate was used as the RNA label (*8*). Thus there is both a DRB-sensitive and a DRB-resistant hnRNA fraction which must be taken

Table 1. Inhibitory effect of DRB (150 μ*M*) on the synthesis of hnRNA in HeLa cells. Triplicate samples of 1 to 2 × 10⁶ cells were used for each determination in spinner medium (*2*, *5*). Treatment with actinomycin D (0.05 μg/ml) was begun 25 minutes before the start of each experiment and continued throughout. In experiments A and B the cells were treated with 150 μ*M* DRB for 15 minutes at 37°C and were then pulsed with [³H]uridine (50 μc/ml) for 15 minutes in the absence (A) or presence (B) of 300 μ*M* uridine (Sigma). In experiment C, [³H]uridine (30 μc/ml) was added to the cells at 4°C for 60 minutes; after washing once with spinner medium the cells were incubated with 150 μ*M* DRB for 5 minutes at room temperature and for 15 minutes at 37°C. At the end of the labeling periods the total cellular and trichloroacetic acid (5 percent TCA)–precipitable radioactivity was determined as described (*2*, *6*).

Condition	[³H]uridine incorporation			
	Total cellular (count/min)	TCA-precipitate		Ratio TCA-prec./total (percent of control)
		Radioactivity (count/min)	Percent of total	
	Experiment A: labeling at 37°C			
Control	5,197,330	190,082	3.7	
DRB	1,010,808	8,803	0.87	24
	Experiment B: labeling at 37°C; 300 μM uridine added			
Control	155,898	6,320	4.1	
DRB	143,411	3,097	2.2	54
	Experiment C: labeling at 4°C			
Control	1,735,525	132,695	7.6	
DRB	1,809,789	48,532	2.7	36

into account in designing experiments to determine the precise mechanism of action of DRB in mammalian cells. To measure the effect of an inhibitor of RNA formation on chain initiation, as opposed to chain elongation, in whole cells, the cells must be exposed to the label for only a brief period so that the incorporated label is predominantly in the nascent RNA chains. As the time of exposure to the initiation inhibitor is increased, the amount of label incorporated will decrease but the chain length of the labeled RNA will increase (*3, 9*). An increase in chain length indicates whether or not chain initiation has been inhibited.

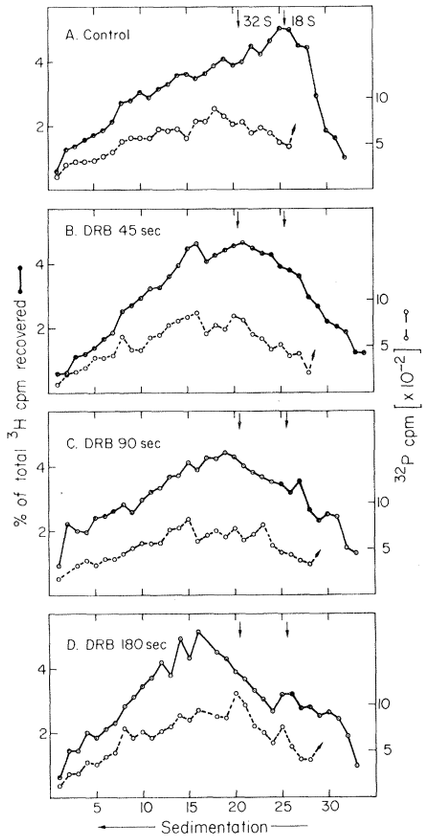

Fig. 1. Effect of prior exposure of cells to DRB on the sedimentation profile of pulse-labeled hnRNA. Cultures of HeLa cells (1×10^8 cells per culture) were treated with actinomycin D (0.04 μg/ml) for 25 minutes in growth medium (*4, 5*) and were then exposed to DRB (75 μM) for 45, 90, or 180 seconds, after which time the cells were labeled with [^{3}H]uridine for 45 seconds (5 mc per 50-ml culture). The cells were fractionated and the nuclei were mixed with nuclei from a culture that had been labeled with 1 to 2 mc of ^{32}P-labeled phosphate (New England Nuclear, carrier-free) for 2 hours in the presence of 0.04 μg of actinomycin D per milliliter (*4, 5*). The hnRNA was extracted, denatured in 80 percent dimethyl sulfoxide as described (*4, 5*), and layered on a 15 to 30 percent sucrose gradient containing 0.01M EDTA, pH 7.4; 0.01M tris, pH 7.4; 0.2 percent sodium dodecyl sulfate; and 0.05M NaCl. The gradients were centrifuged at 20,000 rev/min (SW27.1) for 13 hours. The actual TCA-precipitable ^{3}H radioactivity recovered from each gradient (representing half of each RNA preparation) was 34,956, 9839, 8073, and 6099 count/min in (A), (B), (C), and (D), respectively. After we corrected for the effect of DRB on uridine transport [a factor of about 3 for 75 μM DRB; see Table 1 and (*2*)] these values for (B), (C), and (D) corresponded to 84.4, 69.3, and 52.3 percent, respectively, of the overall rate of RNA synthesis in panel (A).

While the sedimentation profile of hnRNA after a long labeling period shows that maximum labeling occurs in the vicinity of the 32S to 45S fractions (^{32}P profile, Fig. 1, A to D), the profile of hnRNA labeled for extremely short intervals (10 to 45 seconds) shows that labeling occurs mainly in the short molecules (^{3}H profile, Fig. 1A) (*4*). The profile obtained after labeling for short periods has been interpreted as representing the addition of labeled nucleotides to nascent RNA chains (*4*). If DRB selectively blocks chain initiation, then brief exposure of cells to DRB followed by brief exposure to [^{3}H]uridine should lead to an overall increase in the sedimentation rates of nascent chains. Hence, initially there should be a selective inhibition of incorporation of [^{3}H]uridine into the shorter transcripts. The longer the DRB treatment, the longer should be the remaining labeled transcripts.

Cells were treated with DRB for varying times, and exposed to [^{3}H]uridine for 45 seconds; the sedimentation profile of hnRNA was determined as described (*4, 5*). The effect of DRB on uridine transport was eliminated from consideration by comparing the radioactivity (counts per minute) in the sucrose gradient samples as fractions of total acid-precipitable radioactivity recovered, not the incorporated radioactivity per se.

DRB selectively inhibited the labeling of the shorter transcripts, and the mean length of transcripts increased progressively with continued exposure to the drug. Thus DRB selectively inhibited chain initiation (Fig. 1). The total incorporation of [^{3}H]uridine into the faster sedimenting fractions (that is, fractions 2 to 10) was decreased by about 70 percent, but the uptake of [^{3}H]uridine into the cells was decreased by the same amount (data not shown). Thus it seems that DRB did not significantly alter the incorporation of [^{3}H]uridine into the longest transcripts.

The shift in the peak of radioactivity to increasingly longer RNA chains with increasing duration of exposure to DRB (Fig. 1) enabled us to estimate the rate of chain elongation in HeLa cells. We assume that during brief exposure to [^{3}H]uridine the segments that become labeled on the growing chains are of relatively the same length, regardless of the actual sizes of the growing chains. Therefore, the shift in the peak, from chain lengths of about 1000 to 7000 nucleotides in the control (18S to 32S) to chain lengths of 7000 to 16,000 nucleotides after 3 minutes in DRB (32S to 45S), indicates a minimum chain growth rate of about 9000 nucleotides in 3 minutes (50 nucleotides per second). Because DRB may require a minute or so to begin action, hnRNA chain elongation appears to proceed at a rate of 50 to 100 nucleotides per second (Figs. 1 and 2). This value is similar to values for the rate of chain growth of other long RNA molecules (*10*).

By comparing the effects of DRB with those of actinomycin D, which inhibits

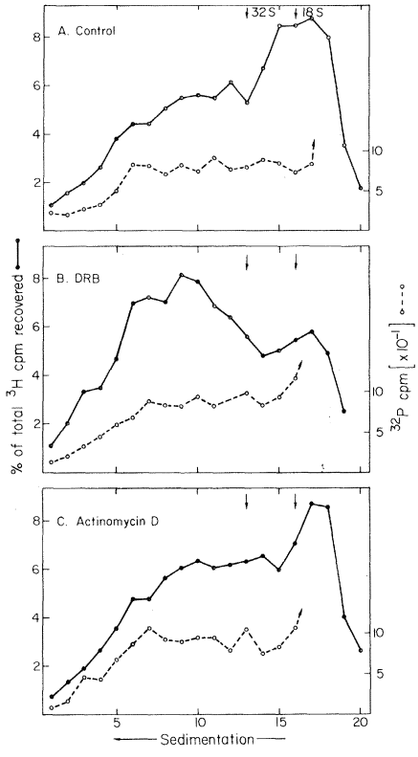

Fig. 2. Effect of prior exposure of cells to DRB or actinomycin D on the sedimentation profile of pulse-labeled hnRNA. The experiment was carried out in a manner similar to that described in Fig. 1. The experimental cultures were treated with DRB (75 μM) for 3 minutes (B) or with actinomycin D (10 μg/ml) for 1 minute (C) and were then pulsed with [^{3}H]uridine (3 mc per culture of 50-ml volume) for 45 seconds. The hnRNA was denatured in dimethyl sulfoxide and the sedimentation profile analyzed by centrifugation at 39,000 rev/min (SW41) for 3.5 hours. The actual TCA-precipitable ^{3}H radioactivity recovered from each gradient (representing half of each RNA preparation) was 29,812 count/min in (A), 3312 in (B), and 9673 in (C). After we corrected for the effect of DRB on uridine transport in this experiment, these values for (B) and (C) corresponded to 49.3 and 32.4 percent, respectively, of the overall rate of RNA synthesis in (A).

chain elongation (*11*), additional support was obtained for the interpretation that DRB shifts the sedimentation profile of hnRNA because it inhibits chain initiation. Cells were treated with a high dose of actinomycin D (10 μg/ml) for 1 minute and then exposed to [^{3}H]uridine for 45 seconds. Although the total amount of ^{3}H incorporated into RNA was decreased by 70 percent, the sedimentation profile of the remaining labeled RNA was similar to that of untreated cells (Fig. 2, A and C). In contrast, treatment with DRB (75 μM) for 3 minutes resulted in a shift (as observed earlier) in the sedimentation profile toward the longer molecules (Fig. 2B).

Because a fraction of hnRNA appears resistant to DRB inhibition [see (*2*) and Table 1] it appears possible that DRB may distinguish between two classes of hnRNA initiated by different RNA polymerases. Other experiments show that the class of hnRNA which is DRB-sensitive contains at least a substantial portion of the molecules which become messenger RNA (mRNA) because no or very little labeled polyadenylate [poly(A)]-containing mRNA reaches the cytoplasm in DRB-treated cells (*8*). Hence, in addition to its value as the only known inhibitor of hnRNA chain initiation in living mammalian cells, it appears possible that DRB may become an important tool in the analysis of the role of RNA polymerases in transcription, and in providing understanding of the regulation of cellular biosynthetic processes (*6*).

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10. For comparison, the rate of RNA chain elongation has been estimated to be at most 100 nucleotides per second for poliovirus RNA [J. E. Darnell, M. Girard, D. Baltimore, D. F. Summers, J. Maizel, in *The Molecular Biology of Viruses*, J. Colter, Ed. (Academic Press, New York, 1967)] and for the 45*S* precursor of ribosomal RNA [H. Greenberg and S. Penman, *J. Mol. Biol.* **21**, 527 (1966)] in HeLa cells. Bacterial RNA polymerases have been estimated to incorporate a maximum of 50 nucleotides per second [H. Bremer and D. Yuan, *J. Mol. Biol.* **38**, 163 (1968)] while the RNA polymerase in *Chironomus tentans* incorporates only about 30 nucleotides per second (*3*).
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12 May 1976

Nuclear and Mitochondrial DNA Replication During Zygote Formation and Maturation in Yeast

Abstract. *Nuclear and mitochondrial DNA replication were monitored during the development of synchronous yeast zygotes. Purified first zygotic buds were also analyzed. Nuclear DNA replicated discontinuously but coincidently with bud initiation, while mitochondrial DNA replicated throughout the zygotic formation and maturation period. First zygotic buds contained the diploid level of both nuclear and mitochondrial DNA.*

Mating is initiated when haploid yeast cells of opposite mating type (*a* and α) are mixed under appropriate conditions. Soon after, cells of opposite mating type pair and, within an hour after cell mixing, clumps of *a* and α cells are evident. One to 3 hours later, after agglutination, *a* and α cell pairs fuse to produce zygotes. After cell fusion, parental cytoplasms mix and parental nuclei fuse. Approximately 0.5 hour after zygote formation, the first zygotic bud is initiated. Genetic analyses of this zygotic bud showed that it contained a diploid nucleus derived from both nuclear parental inputs. In contrast to the stable nuclear genetic composition of zygotic buds, their cytoplasmic genetic constitution can be

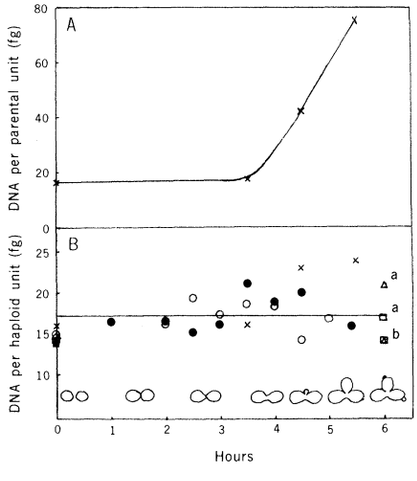

Fig. 1. DNA content during synchronous mating. Haploid single spore isolates *a* 6A *trp-4* and α 6B *trp-4* of diploid *a* 5032A × α 5032B were cultured, mated, and fractionated (*12*). The DNA content of all the samples except the diploid zygotic buds was determined chemically with the DABA method (*4*). Total DNA isolated from 1×10^8 to 2×10^8 diploid zygotic buds was estimated in the analytical ultracentrifuge by measuring the areas under the densitometer tracings relative to a known quantity of marker DNA. All samples taken during the initial 4 hours of mating were removed directly from the mating mixture. After 4 hours, the zygotes were purified (90 to 99 percent) from the nonmating cells. A sample of pure zygotes was taken, and the remainder were inoculated into fresh mating medium for further maturation. Other samples were taken during first zygotic bud formation and after the first zygotic bud was completed. The remaining mature zygotes were fractionated into a first diploid zygotic bud fraction (*b*-released diploid buds represented 10 to 15 percent of the actual formed buds) and a residual zygote fraction (*a*-mature zygotes, 10 to 15 percent of which were without their first zygotic buds) for DNA measurement. The predominant cell morphology during the experiment is diagrammed for reference. [Since cells are not maintained in medium during zygote separation, the time or abscissa positions beyond zygote isolation (4 hours) were determined by comparing the fraction morphology with the age of an unfractionated synchronously mating population.] (A) The kinetics of DNA increase during mating in a typical experiment. Values are calculated per haploid parent. Unbudded cells were counted as one parental unit, while budded cells, unbudded zygotes, and singly and multiply budded zygotes were counted as two parental units. (B) The mean of data points obtained from five synchronous mating experiments (●, ○, ×, △, □). The DNA content per haploid equivalent was calculated by counting unbudded cells as one, budded cells and unbudded zygotes as two, singly budded zygotes as four, doubly budded zygotes as six, triply budded zygotes as eight, unbudded diploid zygotic buds as two, and budded diploid zygotic buds as four. This line through the mean of the data points is not statistically different from the calculated linear regression line.