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Inhibition of Balbiani Ring RNA Synthesis at the Initiation Level

(heterogeneous nuclear RNA/purine nucleoside analogue/RNA chain growth rate)

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ABSTRACT **The nucleoside analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, a selective inhibitor of heterogeneous chromosomal RNA synthesis in salivary gland cells of the midge *Chironomus tentans*, blocks the initiation of transcription in Balbiani rings 1 and 2. The analogue seems to be without appreciable effect on the elongation of growing RNA chains and allows finished molecules to be detached from the chromosomal sites. The RNA chain growth rate, based on measurements of the time required for completion of synthesis of Balbiani ring 1 and 2 RNA molecules after addition of the inhibitor, was estimated to be around 25 nucleotides per second at 18°.**

The nucleoside analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole DRB has been tentatively identified as a selective initiation inhibitor of the synthesis of heterogeneous nuclear RNA (hn RNA) in salivary gland cells of *Chironomus tentans* (1). The sequence of hnRNA events during inhibition by DRB resembled the effect of the initiation inhibitor rifampicin on the bacterial RNA synthesis, but it differed from the inhibitory action exerted by the elongation inhibitor α-amanitin on the synthesis of hnRNA. The results provided support in favor of the initiation-inhibitor hypothesis, but the complexity of the synthesis and processing of hnRNA produced by 90% of the chromosomal material (chromosomes I–III), constituting numerous chromosomal loci, did not allow a straightforward demonstration of the inhibitory effect of DRB. Neither was it possible to decide whether or not the chain growth rate was inhibited.

The Balbiani rings (BRs) on chromosome IV offer, however, a more favorable material for investigation of DRB inhibition. The electrophoretic patterns of BR 1 and BR 2 RNA display asymmetrically distributed radioactivity peaks (2, 3) which probably reflect the simultaneous growth of multiple RNA chains from one or a few related transcription unit(s). The giant RNA transcripts (75S RNA) (3) leave the BR 2 soon after completion and appear in the nuclear sap. Thus, growing RNA chains and the finished product can be separately analyzed after different periods of DRB treatment.

The DRB experiments show a time-dependent, differential suppression of growing BR RNA chains with a preferential inhibition of the shorter RNA chains. The data corroborate the idea that the nucleoside analogue DRB interferes with the transcription of RNA molecules at the initiation level without affecting significantly the elongation rate and completion of nascent molecules. The elongation rate, based on measurement of the time required for completion of BR RNA molecules following addition of DRB, was estimated.

Abbreviations: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; hnRNA, heterogeneous nuclear RNA; BR RNA, RNA from the two major Balbiani rings, BR 1 and BR 2.

MATERIALS AND METHODS

Labeling Conditions. Salivary glands were isolated from fourth instar larvae of the dipteran *Chironomus tentans* (4). In each experiment four animals were used. Four salivary glands were explanted into 50 μl of modified Cannon's medium (5, 6) supplied with 100 μCi of [^{3}H]cytidine at 29 Ci/mmol and 200 μCi of [^{3}H]uridine at 44 Ci/mmol (Amersham) and with DRB (65 μM). The sister glands were used as control glands and the incorporations were carried out at 18° for 20 min. In general, for an appropriate demonstration of the effect of an initiation inhibitor of RNA synthesis, a preincubation period of variable length in combination with a short labeling time is preferable. A drawback to short labeling times is, however, the low degree of incorporation, leading to inaccuracy in the radioactivity measurements. A labeling period of 20 min has been found to be an acceptable compromise and has been used throughout this work.

Isolation of Chromosome IV and Nuclear Sap, and the Extraction of RNA. After incubation the glands were fixed and chromosome IV and nuclear sap were isolated by microdissection (7). The pooled samples of chromosome IV and nuclear sap were then transferred to separate test tubes containing 100 μl of a preincubated solution of 0.02 M Tris·HCl buffer, pH 7.4, sodium dodecyl sulfate (5 mg/ml), and nuclease-free Pronase (1 mg/ml) (Calbiochem), and the samples were digested for 3–5 min at 20–25° (1). After the digestion 30 μg of *Escherichia coli* carrier RNA and 10 μl of 1 M NaCl were added. The material was then precipitated with 2 volumes of absolute ethanol and stored for about 18 hr at 4°.

Electrophoresis and Measurement of Radioactivity. The RNA precipitate was dissolved in 20 μl of a 0.02 M Tris·HCl buffer, pH 8.0, containing 0.02 M NaCl, 2 mM EDTA, and 2 mg of sodium dodecyl sulfate/ml and was then analyzed by electrophoresis in a 1% agarose gel (3). At the end of the run, the gel slabs were treated with cold trichloroacetic acid and finally washed with water (8). They were cut and the slices were transferred to Packard scintillation vials. Ten milliliters of toluene scintillator containing soluene and Permablend (Packard) was added and the vials were incubated for 2.5 hr at 60°. After cooling the radioactivity of the samples was measured in a Packard liquid-scintillation spectrometer at an efficiency of around 33% and at a background of about 15 cpm.

RESULTS

Distribution of labeled RNA from Balbiani Rings 1 and 2 and from the residue of chromosome IV after short labeling time

The electrophoretic analyses of BR 1 RNA, BR 2 RNA, and of RNA from the residue of chromosome IV after 20 min of

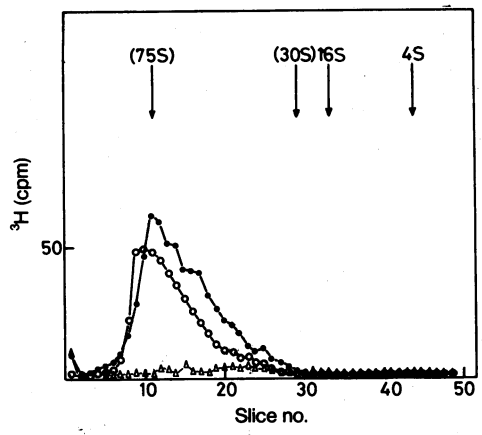

FIG. 1. Electrophoretic separations of labeled RNA from Balbiani rings 1 and 2 and from the residue of chromosome IV after 20 min of labeling. Four glands from two animals were incubated in 50 μl of incubation medium containing 100 μCi of cytidine and 200 μCi of uridine for 20 min at 18°. After incubation the glands were fixed and from each gland six sets of chromosome IV, divided into three fractions (BR 1, BR 2, and the residue), were isolated by microdissection. The labeled RNA from each sample was released by Pronase/sodium dodecyl sulfate treatment, electrophoresis was carried out in 1% agarose gel, and the radioactivity was measured in a Packard liquid-scintillation spectrometer. *E. coli* RNA was used as marker (23S, 16S, and 4S). The position of 30 S was determined in parallel analyses of nucleolar RNA. For more details see *Materials and Methods*. ●, BR 1; ○, BR 2; △, residue of chromosome IV.

incorporation with tritiated nucleosides are shown in Fig. 1. The radioactivity profile of BR 1 RNA mimics that of BR 2 RNA (3). There is a broad and asymmetric peak with maximum activity in slice 10 and 11. The slope on the high-molecular-weight side is steep, whereas it is somewhat variable and less steep on the other side. Non-BR RNA from chromosome IV displays a heterogeneous distribution in the 16–100S range and it comprises less than 10% of the total labeled chromosome IV RNA. However, the relative amounts of BR 1 RNA, BR 2 RNA, and RNA from the residue of chromosome IV vary to some extent. The analyses in Fig. 1 represent average distributions.

Since BR 1 RNA and BR 2 RNA possess similar electrophoretic characteristics, both reflecting spectra of growing RNA chains, and together comprise more than 90% of the total chromosome IV RNA, it was feasible to exclude the laborious dissection work required to carry out separate analyses of BR 1 and BR 2 RNA. For subsequent analyses only RNA from the whole chromosome IV was used.

Residual synthesis and release of RNA from chromosome IV preincubated with DRB for different time periods

(a) Synthesis of RNA

The electrophoretic analyses of chromosome IV RNA after 0, 10, and 15 min of preincubation in DRB followed by 20 min of incorporation with tritiated nucleosides in the continued presence of the inhibitor are shown in Fig. 2a. The control glands were labeled under similar conditions but in the absence of DRB. The migration profile of chromosome IV RNA from control glands after 20 min of labeling displays one main peak with a maximum value in slice 11, with a broad and asymmetric distribution as previously described for

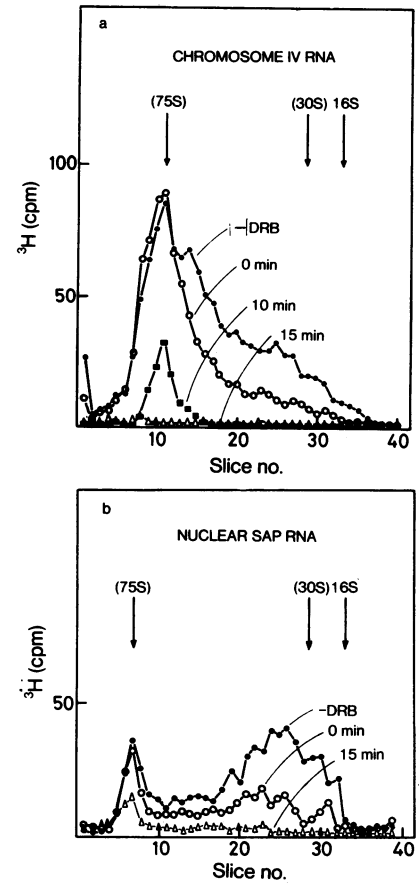

FIG. 2. Electrophoretic separations of chromosome IV RNA (a) and nuclear sap RNA (b) labeled for 20 min in the absence and in the presence of DRB after preincubation in the presence of DRB. Salivary glands were incubated at 18° for 0, 10, and 15 min in 50 μl of incubation medium containing DRB (65 μM). They were then transferred to another 50 μl of the same medium containing DRB, 100 μCi of cytidine, and 200 μCi of uridine and incubated for 20 min. Labeling with tritiated nucleosides in the absence of DRB was carried out in a parallel procedure. All the animals used in these experiments were derived from the same culture. For labeling with isotopic nucleosides without preincubation (● and ○) sister glands were used. Chromosome IV and nuclear sap were dissected from 24 cells (four glands). For other data see the legend to Fig. 1. ●, Labeling in the absence of DRB; ○, labeling in the presence of DRB without preincubation in DRB; ■, labeling in the presence of DRB after 10 min of preincubation in DRB (the profile of nuclear sap RNA is lacking); △, labeling in the presence of DRB after 15 min of preincubation in DRB.

BR 2 RNA (3) and for BR 1 and BR 2 RNA in Fig. 1. A minor 4–5S RNA peak present in the chromosomal fraction is excluded from the figure, as it has been shown not to be sensitive to DRB (1). When DRB was added to the incubation system along with the labeled nucleosides and the glands were incubated for 20 min, the radioactivity profile showed a more distinct and symmetric peak compared to the pattern of normal RNA (Fig. 2a). The level of radioactivity was unchanged in the highest molecular-weight regions of the main peak (75 S), but the incorporation was reduced gradually towards the low-molecular-weight regions. The trailing of the inhibitor profile probably represents a transient escape transcription. This implies that not all of the initiating polymerase molecules are instantly affected by DRB. The labeling

in the 16–50S range was, however, entirely inhibited if the labeled nucleosides were added 10 min after DRB. The symmetric feature of the main radioactivity peak was further accentuated and the labeling of RNA molecules migrating faster than those in slice 20 was completely abolished. The radioactivity in the 75 S range was also reduced but the slightly asymmetric distribution of the main peak indicates that there are still residual RNA chains not completed after 30 min of DRB treatment. The labeling was, however, entirely inhibited if the tritiated nucleosides were added to the glands 15 min after DRB (Fig. 2a). The synthesis of short RNA chains as well as long chains was eliminated. Thus it is evident from Fig. 2a that the later the radioactive precursors were added, the less radioactivity was incorporated into the DRB-sensitive chromosome IV RNA, and further, that the radioactivity disappeared first from the low-molecular-weight regions.

(b) Nuclear sap RNA

Available data strongly suggest that the finished RNA transcripts of BR 2 RNA are released from the chromosomal level into the nuclear sap without measurable size changes (3). The product migrates in the gel as a discrete peak, corresponding to the 75S RNA in BR 2. The electrophoretic pattern of nuclear sap RNA after 20 min of incubation with isotopic nucleosides exhibits a bimodal distribution: there is a rather distinct 75S peak and label heterogeneously distributed in the 16–50S range (Fig. 2b). The chromosomal site of the synthesis of this heterogeneous 16–50S RNA is not yet established, but an origin from puffs other than BR 1 and BR 2 seems very likely. If the mode of action of DRB on the synthesis of BR RNA is that postulated, i.e., an inhibition of initiation but not of elongation, one would expect in the presence of inhibitor a normal flow rate for the 75S RNA from the chromosomal sites to nuclear sap, at least during the first 20 min of labeling period. This is because the radioactivity level in the 75S region of the chromosome IV RNA remains essentially unaffected after 20 min of drug treatment. The expected result is seen in Fig. 2b. After a coincident addition of DRB and labeled nucleosides the glands were incubated for 20 min and the nuclear sap RNA was analyzed. The amount of labeled RNA released to the nuclear sap was diminished by more than 60% in the 16–50S region compared with the profile of normal nuclear sap RNA, but the amount of the released 75S RNA was not influenced significantly. When the labeled nucleosides were added 15 min after DRB, the electrophoretic pattern of nuclear sap RNA no longer contained an appreciable amount of labeled RNA in the 16–50S region, but labeled 75S RNA was still entering the nuclear sap, although to a reduced extent.

Regression of Balbiani Ring 1 and 2 in the presence of DRB

Although the mechanism behind the appearance of puffs like BRs in polytene chromosomes from dipteran salivary glands is not yet understood, there is experimental support for some correlation between RNA synthesis and puffing (9, 10). Studies with different antimetabolites which affect RNA synthesis and puffing in explanted salivary gland cells of *Chironomus* indicated a dependence of RNA synthesis on the puffing, but the puffing seemed not to depend on RNA synthesis (9). When the salivary glands were treated with DRB

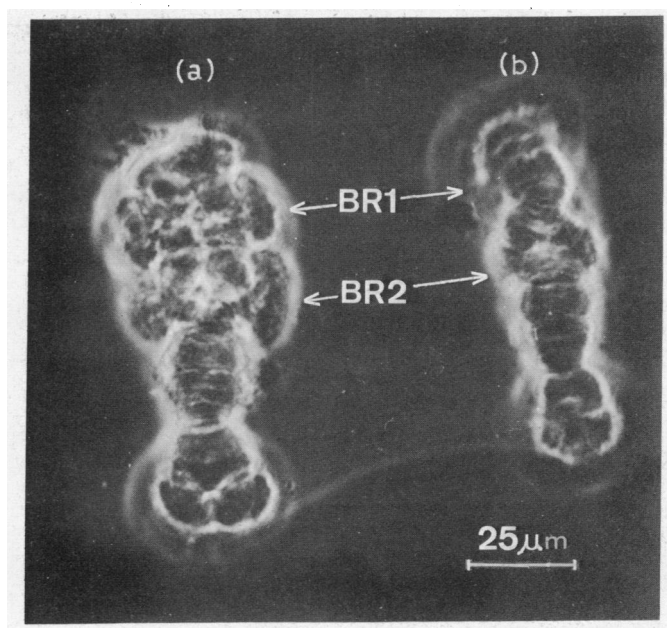

FIG. 3. Photomicrograph of isolated chromosomes IV after incubation with DRB for 30 min (a) and 40 min (b). Two sister glands were incubated in 25 μl of incubation medium containing DRB (65 μM) at 18°. After 30 min of incubation one gland was removed from the incubation droplet and was fixed. The incubation of the remaining sister gland was continued for an additional 10 min before fixation. The chromosome IV was isolated by microdissection. The bar represents 25 μm.

for 30 min, no difference in size between treated and untreated BRs could be established (data not shown). If, however, the incubation with the inhibitor was prolonged from 30 to 40 min, a dramatic size reduction of BRs could be observed (Fig. 3). These experiments indicated that as long as RNA synthesis is taking place in the BRs, even if at a decreased rate, the puffs preserved roughly their original size. However, simultaneously with or soon after termination of RNA synthesis an extensive shrinkage of BR 1 and 2 was apparent. Thus, the results provide further support in favor of a correlation between RNA synthesis and puffing, and accord with the possibility that puffing may depend on RNA synthesis.

DISCUSSION

DRB inhibits the initiation but not the rate of elongation

The data presented here demonstrate a time-dependent, differential inhibition of the synthesis of BR RNA by DRB, with a preferential suppression of labeling in the lower-molecular-weight regions, in accordance with previous studies of hnRNA (1). However, in the present work the experimental situation has been improved considerably by studying RNA from only a few defined chromosomal regions, containing similar transcription units. The fact that the BR 1 and the BR 2 are producers of giant RNA transcripts which both have similar electrophoretic properties enabled an extensive spectrum of nascent BR RNA chains to be analyzed at increasingly longer times after DRB addition. Accordingly, the preferential inhibition of smaller RNA molecules could not be caused by a selective inhibition of transcription of smaller genes. The finding that after 30–35 min of drug treatment the labelings of small and large molecules are equally

abolished speaks also against such an explanation. The accumulated data support the idea that DRB interferes with the hnRNA transcription at the initial level. The main arguments presented in this report may be summarized in two points. First, the largely asymmetric profile of BR RNA becomes gradually symmetric when DRB and tritiated nucleosides are added coincidently or when the isotopic nucleosides are added at progressively later times after DRB. Second, 20 min after a coincident addition of DRB and radioactive precursors, the radioactivity profile of BR RNA shows a marked reduction in the low-molecular-weight range but remains unaffected in the 75S region, and finished 75S RNA molecules appear in the nuclear sap at a normal rate during the first 20 min of DRB treatment. An uninterrupted flow of 75S RNA to the nuclear sap implies also that the nucleoside analogue does not influence appreciably the elongation rate of BR RNA.

Determination of chain growth rate

After administration of an inhibitor of transcription initiation there is a lag period until the RNA synthesis is terminated. This is due to the fact that RNA chains already initiated when the inhibitor is added (nascent chains) will be completed. Consequently, this delay time should be equal to the time required for an RNA polymerase molecule to transcribe the whole transcription unit. The time after addition of DRB at which isotopic nucleosides can no longer be incorporated into the BR RNA should therefore, according to the present data, correspond to the synthesis time of BR RNA. The electrophoretic analyses from DRB-treated cells indicate a time period of 30–35 min for a complete suppression of BR RNA. Such an estimate is, however, beset with uncertainties and a more exact figure in these studies could not be obtained due to the following reasons. First, the time lapse between administration of inhibitor and inhibition of transcription is not exactly known, but should not exceed 5 min (unpublished observations). Second, the time after DRB addition at which BR RNA no longer incorporated labeled precursors was, according to the electrophoretic analyses, longer than 30 min but shorter than 35 min, a more precise determination being difficult to achieve in this experimental system. Third, accumulation of finished BR RNA on the chromosomes may have prolonged falsely the estimation of synthesis time. An accumulation of finished RNA transcripts for a shorter period cannot be excluded, although the duration for a presumed accumulation should not exceed 5 min, as indicated by the results in Fig. 2a. After 30 min of DRB treatment the pattern of BR RNA still had, to a certain extent, an asymmetric nature, indicating the existence of residual unfinished RNA chains. When the time after DRB was prolonged by 5 min, the treatment was, however, sufficiently long for all the residual RNA molecules to be completed and released into the nuclear sap. Each of the three mentioned sources of inaccuracy may have influenced the estimate in the same direction, leading to an overestimation of the synthesis time. It is, therefore, conceivable that the synthesis time of BR RNA lies closer to 30 than to 35 min.

For the determination of chain growth rate the molecular weight of finished BR RNA transcripts has to be known. Although the molecular weight determination of such large molecules is beset with considerable difficulties, the molecular weight of BR 2 RNA seems to be in the range of 15 million (11). This figure, together with the estimated synthesis time of 30 min for BR RNA, indicates a chain growth rate of approximately 25 nucleotides per second. There are no comparable data available on the elongation rate of hnRNA in other eukaryotic cells. Estimates of mRNA elongation rate in *Escherichia coli* include a wide range of values (12), but some of the latest reported estimates, obtained by independent methods, show a good agreement (13, 14). The elongation rate for mRNA of the tryptophan operon was calculated to be 37 to 45 nucleotides per second at 37° and 16 to 17 nucleotides per second at 25°. In view of the estimated chain growth rate of approximately 25 nucleotides per second for BR RNA at 18°, the elongation rates of bacterial mRNA and eukaryotic BR RNA are in reasonable agreement.

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1. Egyházi, E. (1974) *J. Mol. Biol.* **84,** 173–183.
2. Daneholt, B., Edström, J. -E., Egyházi, E., Lambert, B. & Ringborg, U. (1969) *Chromosoma* **28,** 418–429.
3. Daneholt, B. (1972) *Nature New Biol.* **240,** 229–232.
4. Beermann, W. (1952) *Chromosoma* **5,** 139–198.
5. Cannon, G. B. (1964) *Science* **146,** 1063.
6. Ringborg, U. & Rydlander, L. (1971) *J. Cell Biol.* **51,** 355–368.
7. Edström, J. -E. (1964) in *Methods in Cell Physiology,* ed. Prescott, D. (Academic Press, New York), Vol. 1, pp. 417–447.
8. Egyházi, E. (1974) *Nature* **250,** 221–223.
9. Beermann, W. (1967) in *Heritage from Mendel,* ed. Brink, R. A. (Univ. Wisconsin Press, Madison, Wisc.), pp. 179–201.
10. Berendes, H. D. (1973) *Int. Rev. Cytol.* **35,** 61–116.
11. Daneholt, B. (1975) *Cell* **4,** 1–9.
12. Geiduschek, E. P. & Haselkorn, R. (1969) *Annu. Rev. Biochem.* **38,** 647–676.
13. Manor, H., Goodman, D. & Stent, G. S. (1969) *J. Mol. Biol.* **39,** 1–29.
14. Rose, J. K., Mosteller, R. D. & Yanofsky, C. (1970) *J. Mol. Biol.* **51,** 541–550.