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Induction of premature termination of transcription of the mouse β-globin gene by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)

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ABSTRACT

Hybridization of pulse-labeled RNA from DRB-treated Friend cells to mouse β-globin cDNA revealed that the appearance of β-globin mRNA in the cytoplasm was inhibited by greater than 87°/•. To examine the effect of DRB (125 μM) on HnRNA synthesis, nuclear RNA was electrophoresed in methyl mercuric hydroxide gels, transferred to nitrocellulose, and hybridized with β-globin specific probes. Full-length nuclear transcripts, while present in untreated cells, were not detected in DRB-treated cells. Using restriction enzymes, the cloned β-globin gene was divided into fragments proceeding from the 5' gene region to the 3' gene region. RNA labeled in vitro by transcription in nuclei isolated from DRB-treated cells hybridized only to the promoter proximal DNA fragment. Transcripts hybridizing to fragments from both the 5' and 3' regions of the gene were produced in nuclei from untreated cells. Together these results indicate that DRB causes premature termination of transcription within the β-globin gene.

INTRODUCTION

Several studies have demonstrated the usefulness of DRB in analyzing the regulation of transcription in eukaryotic cells (1-4). This drug, a halogenated analog of adenosine, selectively and reversibly inhibits approximately 70°/$_{o}$ of heterogeneous nuclear RNA (HnRNA) synthesis in human, murine, avian, and insect cells and blocks the appearance of mRNA in the cytoplasm by $\geq$ 95°/$_{o}$ (2, 5, 6). Examination of adenovirus and simian virus 40 (SV 40) transcriptional units has revealed that DRB causes early termination of RNA synthesis resulting in the accumulation of short RNA chains transcribed from the promoter proximal regions of the viral genomes (7-10). DRB may similarly block transcription of cellular RNAs at some point downstream from the site of initiation. RNA chains ranging from approximately 100-500 nucleotides in length have been shown to accumulate in DRB-treated HeLa cells (11-13).

The finding that short HnRNA transcripts were also generated in HeLa cells and Chinese hamster ovary cells not treated with DRB (11, 14) has led

to the speculation that premature termination of transcription may be a normal cellular process. Such transcription attenuation may be characteristic of eukaryotic systems since partial viral transcripts have also been observed, in the absence of DRB, during adenovirus and SV 40 infection (10, 15). Addition of DRB enhances the premature termination such that no complete transcripts are made.

Whether DRB acts directly or indirectly with putative physiological attenuation regions to accentuate transcription termination has not been determined. To evaluate further the mechanism and site of action of DRB on synthesis of RNAs, we have studied its effect on transcription of a specific cellular HnRNA, β-globin HnRNA, in Friend erythroleukemic cells. Because the β-globin gene has been cloned and extensively characterized (16-18), probes specific for defined regions of the gene could be prepared (Fig. 1). With these probes the extent of transcription in the presence of DRB was determined and the approximate region of the gene in which DRB-induced termination occurs was identified.

MATERIALS AND METHODS

Cell culture. Friend erythroleukemic cells, clone 745, were propagated in Ham's F-12 medium (Flow Lab.) supplemented with 10°/₀ fetal calf serum. Cultures at 1 x 10^5 cells/ml were induced to differentiate for 72-84 h by addition of dimethyl sulfoxide to 1.8°/₀. For DRB treatment, cells were concentrated to 5 x 10^6 cells/ml and DRB was added from a stock solution of 20 mM DRB in ethanol to a final concentration of 125 μM. Untreated cells received an equivalent amount of ethanol.

Isolation of RNA. To isolate cytoplasmic RNA, cells were poured over

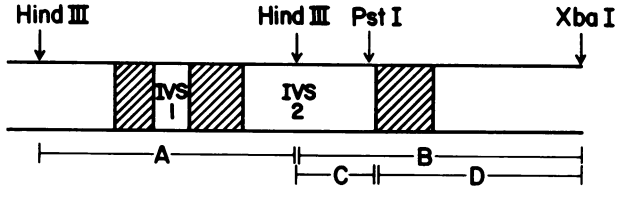

Figure 1. Restriction map of the cloned mouse β-globin gene (18). The first (IVS 1) and second (IVS 2) intervening sequences and the β-globin structural sequences (shaded areas) are indicated. Restriction fragments A and B were prepared by digestion of pBR322·β62 with Hind III and Xba I followed by electrophoresis on agarose gels. Fragment B was further restricted with Pst I to generate fragments C and D.

crushed frozen medium, collected by centrifugation and washed with 0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl (containing 125 μM DRB for DRB-treated cells). Cells were lysed and the cytoplasmic fraction recovered as described previously (19). This fraction was adjusted to 0.5°/₀ sodium dodecyl sulfate (SDS), 0.01 M EDTA and the RNA was extracted three times with buffer (0.1 M Tris-HCl, pH 9.0)-saturated phenol:chloroform:isoamyl alcohol (50:49.5:0.5), two times with chloroform:isoamyl alcohol, and ethanol precipitated.

To isolate nuclear RNA, nuclei were prepared as previously described (20). The nuclear pellet was resuspended in buffer containing 0.02 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 M NaCl, 3°/₀ SDS, and 300 μg/ml proteinase K. After incubation at 37°C for 30 min, the suspension was extracted three times with phenol:chloroform:isoamyl alcohol and two times with chloroform:isoamyl alcohol. After ethanol precipitation the nuclear RNA was incubated with iodoacetate treated DNAase (21) and phenol:chloroform extracted as described (22).

In vitro labeling of RNA in isolated nuclei. Nuclei were isolated by lysis of cells with 0.5°/₀ Triton X-100 as previously described (19). Nuclei were resuspended in 0.5 ml of the cell-free transcription buffer of Laub et al. (10) containing 0.5 mM ATP, CTP, and GTP and 0.2 mCi of [α-^{32}P] UTP (300-600 Ci/mmole, Amersham). Incubation was at 26°C after which RNA was extracted as described (22).

Plasmid production and restriction endonuclease digestion. Plasmids pBR322·βG2 and pCR1 βcDNA were grown in HB101 and C600rK$^-$,mK$^-$, respectively, in minimal medium supplemented with 100 μg/ml ampicillin (HB101) or 20 μg/ml kanamycin (C600rK$^-$,mK$^-$). After growth to an absorbance of 0.6, chloramphenicol was added to 200 μg/ml and incubation at 37°C was continued for 18 h. DNA isolation and centrifugation in cesium chloride-ethidium bromide gradients was as described by Clewell and Helinski (23) except that the detergent used in the lytic mixture was 0.2°/₀ Triton X-100. Restriction fragments were prepared by cleavage of pBR322·βG2 with Xba I and Hind III (18), and were separated by electrophoresis in 1.8°/₀ agarose gels (24). β-globin gene fragments were recovered from gel slices by electroelution into dialysis bags at 50 mA for 16 h. The DNA was purified over DEAE-cellulose columns (25) and further digested with Pst I.

Nick translation. DNA was labeled by nick translation to a specific activity of 1.2 x 10^8 cpm/μg. Reactions were performed in a final volume

of 100 µl containing 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.5 µg bovine serum albumin, 1 µg DNA, 0.02 mM dATP, dGTP, dTTP and 125 µCi [α-^{32}P] dCTP (400-600 Ci/mmole, Amersham). After addition of 7.5 U E. coli DNA polymerase I and 150 pg DNAase I, the mixture was incubated for 2 h at 14°C. The reaction was stopped by addition of EDTA to 0.01 M followed by phenol-chloroform extraction and ethanol precipitation.

Gel electrophoresis of RNA. RNA samples were separated by electrophoresis on horizontal slab gels containing 1.5°/$_{\circ}$ agarose and 10 mM methylmercuric hydroxide using the buffer system of Chandler et al. (26). Samples were denatured for 20 min at room temperature with 10 mM methyl mercuric hydroxide in a two-fold dilution of buffer. Glycerol was added to 10°/$_{\circ}$ and electrophoresis was at 80 V for 4 h. Gels were prepared for transfer to nitrocellulose using the modified procedure of Alwine et al. (27).

Blotting of RNA and DNA and hybridization to probes. Preparation of diazobenzyloxymethyl (DBM)-paper and transfer of DNA from agarose gels to the paper was as described by Wahl et al. (28). After transfer the paper was pretreated with buffer containing 50°/$_{\circ}$ formamide, 0.75M NaCl, 0.075M sodium citrate, 29 mM N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES), pH 7.5, 0.1°/$_{\circ}$ each of bovine serum albumin, ficoll, and polyvinyl pyrrolidone, 1°/$_{\circ}$ glycine, 0.1°/$_{\circ}$ SDS, and 0.25 mg/ml yeast tRNA for 5 h at 43°C. Hybridization with ^{32}P-RNA synthesized in vitro was in the same buffer except ficoll, bovine serum albumin and polyvinyl pyrrolidone were at 0.02°/$_{\circ}$. Hybridization was at 43°C for 23 h. Blots were washed according to Meyuhas and Perry (29), treated with 10 µg/ml pancreatic RNAase and 10 U/ml TI RNAase, and exposed to X-ray film at -70°C using Kodak (Cronnex Hi-plus) intensifying screens.

RNA was transferred from agarose gels to nitrocellulose by the method of Thomas (30). Prehybridization, hybridization with nick translated probes, and washing of the filters were done as described by Wahl et al. (28) except that prehybridization and hybridization buffers contained 29 mM TES, pH 7.5, and 0.1°/$_{\circ}$ SDS.

Hybridization of ^{3}H-RNA to DNA filters. Plasmid pCR1·βcDNA was linearized by Hind III digestion (31) and loaded on BA85 nitrocellulose filters (Schleicher and Schuell) as described (32). The filters were air-dried for 1 h and baked in a 60°C vacuum oven overnight. The filters were incubated in 0.02°/$_{\circ}$ each of ficoll, bovine serum albumin, and polyvinyl pyrrolidone in 0.6M NaCl, 0.06M sodium citrate for 1 h at room

temperature and baked in a 70°C vacuum oven for 4 h. Hybridization of ^{3}H uridine-labeled RNA to the DNA filters was in 0.5M NaCl, 0.01M Tris-HCl, pH 7.5, at 66°C for 24 h. Hybridization reactions contained a volume of 200 µl and were covered with paraffin oil. Filters were washed two times with 0.30M NaCl, 0.03M sodium citrate (2 x SSC) at 50°C for 20 min and one time at room temperature. Filters were treated with 10 µg/ml pancreatic RNAase in 2 x SSC for 30 min at 37°C, washed with 2 x SSC, dried, and radioactivities determined.

RESULTS

Effect of DRB on appearance of β-globin RNA in cytoplasm. The effect of DRB on β-globin mRNA was investigated by pretreating dimethyl sulfoxide-induced Friend cells with 125 µM DRB for 30 min and then labeling with ^{3}H-uridine in the presence of the drug. At 60, 90, and 120 min after addition of the label, aliquots of cells were harvested and the cytoplasmic RNA extracted. The incorporation of ^{3}H-uridine into β-globin mRNA was then measured by hybridization of the RNA to nitrocellulose filters containing 4 µg of denatured β-globin cDNA. As shown in Fig. 2, DRB inhibited the appearance of β-globin specific sequences in the cytoplasm by greater than 87%. Whereas β-globin mRNA accumulated linearly with time in untreated cells, only low levels of β-globin RNA were detected in

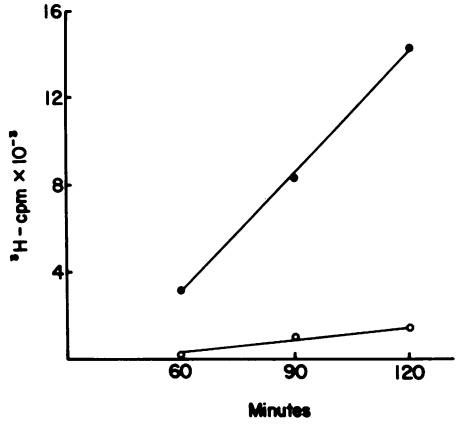

Figure 2. Effect of DRB on the accumulation of β-globin mRNA in the cytoplasm. After pretreatment for 30 min with 125 µM DRB, cells were pulsed with ^{3}H-uridine. At the indicated times, cytoplasmic RNA was extracted and β-globin specific mRNA was measured by hybridization to filters containing β-globin cDNA. (●) untreated cells; (○) DRB-treated cells.

DRB-treated cells. Interestingly, these results did not have to be corrected for an inhibitory effect of DRB on transport of nucleosides into the cells. Unlike other cell systems (2, 5), no significant differences were observed in ^{3}H-uridine uptake between untreated and DRB-treated Friend cells (data not shown). It is possible that alterations in membrane permeability which accompany Friend cell differentiation may account for this effect (33).

Effect of DRB on β-globin HnRNA. The substantial reduction in the amount of labeled β-globin mRNA present in the cytoplasm of DRB-treated cells indicated that DRB may be blocking either the processing or transcription of β-globin HnRNA. To resolve this question, unlabeled nuclear RNA from untreated and DRB-treated (125 μM for 60 min) cells was isolated and approximately 20 μg was electrophoresed in agarose slab gels containing 10 mM methyl mercuric hydroxide. Following transfer of the RNA to nitrocellulose filters, the presence of β-globin sequences was measured by hybridization to ^{32}P-labeled fragment A (Fig. 1) of the globin gene. Autoradiography of the blots revealed that, in untreated cells, two species of RNA hybridized to the nick translated probe (Fig. 3c). The predominant species had an electrophoretic mobility similar to that of purified 9S

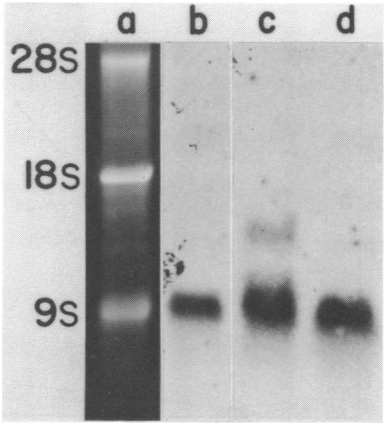

Figure 3. Hybridization of nuclear RNA from untreated and DRB-treated (125 μM for 60 min) cells with ^{32}P-β-globin probe. Nuclear RNA was extracted and electrophoresed in methyl mercuric hydroxide gels. After transfer to nitrocellulose, the RNA was hybridized with nick-translated β-globin fragment A. (a) 9S β-globin mRNA, 28S and 18S ribosomal RNAs; (b) Cytoplasmic RNA from untreated cells; (c) Nuclear RNA from untreated cells; (d) Nuclear RNA from DRB-treated cells.

β-globin mRNA (Fig. 3a) and was present both in nuclear and cytoplasmic fractions (Fig. 3b and c). The second species was observed only in nuclear RNA preparations (Fig. 3c) and had a molecular weight corresponding to that of the 15S β-globin mRNA precursor (34, 35). As can be seen in Fig. 3d, no sequences corresponding in size to the full length β-globin transcript were detected in nuclear RNA from DRB-treated cells. Only β-globin sequences migrating in the 9S or less region of the gel were present in these cells.

If DRB is affecting the β-globin transcriptional unit by inducing premature termination, the recently synthesized nuclear β-globin sequences in DRB-treated cells should represent promoter proximal sequences (7-11). To determine if this was the case, dimethyl sulfoxide-induced Friend cells were pretreated for 30 min with 125 μM DRB. Nuclei were isolated and treated for 10 min on ice with heparin (2 mg/ml) to inhibit RNA chain initiation and to enhance detection of in vitro elongated transcripts (12, 36). It has been shown previously with nuclei from HeLa (12) and SV 40 infected monkey cells (10) that RNA polymerases can elongate in vitro the DRB-induced, prematurely terminated RNA chains. Nuclei were then incubated at 26°C in the presence of ^{32}P-UTP. At 3 and 12 minutes after addition of the label, RNA was extracted and hybridized to a DBM-paper blot containing β-globin fragments A, C, and D. Fragment A contains a 350 base pair region immediately preceding the cap site followed by the first 690 base pairs of the β-globin gene. The 380 base pairs immediately downstream are localized entirely within the large intervening sequence and are contained in fragment C. The remaining approximately 500 base pairs of the gene are present in fragment D. The RNA synthesized within 3 min in nuclei isolated from DRB-treated cells hybridized exclusively to fragment A (Fig. 4f and g). Even after 12 min of in vitro synthesis, hybridization of RNA from DRB-treated cells was restricted to this fragment (Fig. 4h and i). In contrast, in nuclei from untreated cells, the RNA synthesized after 3 and 12 min incubations in vitro hybridized to fragment D in addition to fragment A (Fig. 4b-e). Similar results were obtained when nuclear RNA preparations from untreated and DRB-treated cells were transcribed in vitro in the absence of heparin (data not shown). The majority of the RNA hybridizing to fragment A can be classified as β-globin specific since Hofer and Darnell (37) have recently shown that at least 80°/$_{o}$ of the RNA transcribed from the β-globin region in vitro in Friend cell nuclei had been initiated at the cap site. Thus, these observations indicate that addition of DRB to differentiating Friend cells results in the premature termination

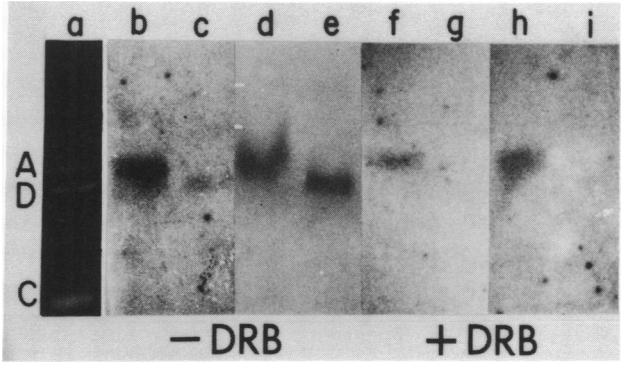

Figure 4. In vitro transcription of β-globin HnRNA in nuclei isolated from untreated and DRB-treated cells. After pretreatment of cells for 30 min with 125 μM DRB, nuclei were isolated, treated with heparin (2 mg/ml) and incubated in the presence of [α-^{32}P] UTP for 3 (Lanes b, c, f, g) or 12 (Lanes d, e, h, i) min. RNA was extracted and hybridized to DBM blots containing β-globin fragments A and C (Lanes b, d, f, h) and fragment D (Lanes c, e, g, i). Lane a shows an ethidium bromide-stained gel in which β-globin fragments A, C, and D have been electrophoresed.

of β-globin gene transcription. Whereas RNA sequences hybridizing to the promoter proximal gene region (fragment A) were transcribed in the presence of DRB, RNA sequences homologous to regions of the β-globin gene distal to the promoter (fragment D) were not detected.

The lack of hybridization of RNA, transcribed in nuclei from DRB-treated cells, to fragment C suggested that the site of premature termination was upstream from these sequences. The fact that such hybridization was not observed in heparin-treated nuclei, in which transcripts would be expected to have elongated 300-400 nucleotides (12), points to an in vivo block by DRB at a site within fragment A. Interpretation of this result was difficult, however, because the amount of RNA, synthesized in vitro in nuclei from untreated cells, which hybridized to fragment C was greatly reduced compared to that which hybridized to fragments A and D. Whereas this may be due to processing, in isolated nuclei, of RNA corresponding to β-globin intervening sequences, inefficiency in the hybridization to fragment C or transfer of fragment C to DBM-paper could not be ruled out. To resolve these possibilities, RNA transcribed in vitro was hybridized to DBM-paper containing β-globin fragments A and B. Since fragment B encompasses fragments C and D (Fig. 1), RNA transcribed in nuclei isolated from DRB-treated cells which hybridizes to it should

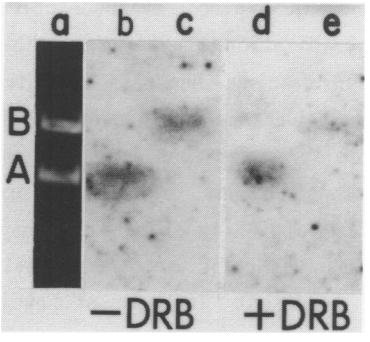

Figure 5. Hybridization of RNA transcribed in vitro in nuclei isolated from untreated and DRB-treated cells to β-globin DNA. DRB pretreatment and nuclei isolation were as described in legend to Fig. 4. Nuclei were incubated in the presence of [α-^{32}P] UTP for 12 min. RNA was extracted and hybridized to DBM blots containing β-globin fragments A (Lanes b and d) and B (Lanes c and e). Lane a shows an ethidium bromide-stained gel in which fragments A and B have been electrophoresed.

represent sequences complementary to fragment C. Fig. 5 shows that RNA synthesized for 12 min in nuclei from untreated cultures hybridized extensively to both fragments A and B. In contrast, RNA transcribed in nuclei from DRB-treated cells hybridized primarily with fragment A. The amount of ^{32}P-RNA which hybridized to fragment B was significantly reduced.

DISCUSSION

These results constitute the first demonstration of premature termination of the nuclear transcript of a specific cellular gene, for β-globin, by DRB. Although the termination of cellular transcription by DRB was suggested by earlier studies, in which the accumulation of short, capped HnRNA chains in DRB-treated HeLa cells was observed (11-13), no direct evidence for premature termination of a specific cellular transcriptional unit was presented. The availability of cloned, highly characterized cellular genes has now made such a study possible because sensitive probes representing defined regions of a gene could be prepared for use in hybridization. In this paper, two lines of evidence pointing to the premature termination of transcription of the mouse β-globin gene in the presence of DRB were obtained. Hybridization of nick translated β-globin DNA probes to nuclear RNA which had been denatured and separated in agarose gels revealed that transcripts corresponding in size to that of the 15S β-globin precursor were not detected in DRB-treated cells (Fig. 3). These

results indicated a block in β-globin transcription was induced by DRB. This was confirmed by hybridization of RNA transcribed in vitro in isolated nuclei to DNA fragments containing sequences from the 5' or 3' regions of the β-globin gene. The RNA transcribed in nuclei from DRB-treated cells hybridized exclusively to promoter proximal sequences (Figs. 4 and 5). Together, these observations account for the failure of β-globin mRNA to accumulate in the cytoplasm of DRB-treated cells (Fig. 2) and indicate that the low levels of β-globin specific sequences that were present in the cytoplasm most likely represent leakage of nuclear RNA during cell fractionation.

The sensitivity of the β-globin transcriptional unit to DRB resembles that observed for adenovirus and SV40 DNA (7-10). In the viral systems, however, DRB appears to enhance a premature termination process which limits the extent of viral transcription in the absence of DRB (10, 15). A similar effect of DRB on HeLa cell HnRNA transcription termination has been documented (11, 12), suggesting that termination of transcription within transcriptional units may be a transcriptional control mechanism utilized by eukaryotes. The present report provides preliminary evidence that premature termination of β-globin HnRNA may occur in the absence of DRB. This indication comes from analysis of the RNA transcribed in vitro (at 26°C) during a brief (3 min) pulse with [^{32}P] UTP of nuclei isolated from cells not treated with DRB. Fig. 4b and c shows that the relative amount of labeled RNA hybridizing to the promoter proximal fragment A is higher than that hybridizing to the more distal fragment D. This difference may reflect a higher level of transcription from β-globin DNA sequences near the initiation site relative to the rest of the gene. Hofer and Darnell (37) recently reported equimolar binding of Friend cell nuclear RNA throughout a set of β-globin DNA fragments ranging from the cap site to beyond the site of Poly(A) addition. The RNA utilized in these experiments, however, was transcribed in vitro (at 37°C) in nuclei during a much longer (15 min) pulse, after which detection of short, nascent RNA chains is difficult (10). Further comparisons of the amounts of β-globin HnRNA corresponding to various gene regions produced both in differentiating and nondifferentiating Friend cells is needed to determine if termination signals are present within this gene and whether their function is altered during gene activation. Because of its sensitivity to DRB, the β-globin transcriptional unit is a potential system for defining the relationship between the region of DRB-induced termination and putative physiological termination sites.

Although the specific site of DRB-induced termination has not been identified, the in vitro transcription results suggest that β-globin transcription is inhibited approximately within the first 500 nucleotides (Fig. 5). β-globin transcripts, labeled in nuclei isolated from DRB-treated cells, hybridized primarily to the promoter proximal fragment A. During the 12 min incubation in vitro, transcripts would be expected to have elongated 300-400 nucleotides (12). The low level of hybridization observed to fragment B indicates that elongation of transcripts into this region of the β-globin gene occurred to only a limited extent. Thus, the majority of synthesis appeared to be localized upstream within fragment A. DRB-induced termination within this gene region would result in the accumulation of short transcripts within the size range of those observed in adenovirus and SV40-infected cells in the presence of DRB (8, 10). Substantial amounts of β-globin RNA sequences of approximately 9S were present in nuclear RNA extracted from DRB-treated cells (Fig. 3). Whereas some of these sequences may be due to mature 9S β-globin mRNA that has not been transported to the cytoplasm or to cytoplasmic contamination, many may represent prematurely terminated, promoter proximal sequences. SI nuclease mapping with β-globin gene fragments, end-labeled within the intervening sequences, should be useful in examining these possibilities and in more precisely defining the gene region in which the termination occurs.

Recent information suggests that specific nucleotide sequences may be control signals for transcription and processing of RNA in eukaryotes (38-40). The features of possible termination sites present within eukaryotic transcriptional units remain to be elucidated. In SV40 DNA, a stretch of nucleotides near the region of DRB-induced transcription termination shares some sequence and structural similarities with bacterial attenuation sites (41, 42). Examination of the terminal regions of β-globin transcripts generated in the presence of DRB should provide insight into the base sequences contributing to the DRB-sensitive site. As the effect of DRB on other cloned cellular genes is investigated, comparisons of gene sequences and structure near regions of termination can be made and possible terminator sequences identified.

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pCR1•βcDNA, respectively. The help and advice of Dr. S. Tilghman (Institute for Cancer Research at Fox Chase) and co-workers in preparation of plasmid DNA is greatly appreciated.

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