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α-Amanitin-insensitive transcription of mouse β^{major}-globin 5′-flanking and structural gene sequences correlates with mRNA expression

(erythroleukemia cells/gene expression/*in vitro* transcription)

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ABSTRACT **A small proportion of the RNAs of mouse reticulocytes consists of β^{major}-globin mRNA sequences linked to sequences transcribed from the 5′-flanking region of the β^{major}-globin gene. These upstream RNAs are polyadenylylated and contain 700–800 nucleotides, and their 5′ regions are heterogeneous. RNAs with similar or identical 5′ regions are transcribed in cell-free extracts from a circular mouse β^{major}-globin gene template. Synthesis of most of the upstream RNAs *in vitro* is not inhibited by low levels (1 μg/ml) of α-amanitin, indicating that they are transcribed by an enzyme(s) different from RNA polymerase II. During culture of mouse erythroleukemia cells with dimethyl sulfoxide, globin mRNA and upstream RNAs accumulate with similar kinetics. In contrast, upstream RNAs are not detected in hemin-treated cells.**

RNAs produced from some structural genes in eukaryotic cells are heterogeneous at their 5′ or 3′ termini (or both). For example, most uteroglobulin, ovalbumin, and human globin RNA molecules begin at the cap site, but a minority contains extra nucleotides that are transcribed from sequences located 5′ to the cap site (1–7). No function has been ascribed to these so-called upstream RNAs. Recently, we demonstrated that upstream RNAs are generated from a circular human β-globin gene template in a cell-free transcription system (6). As determined by S1 nuclease and primer extension assays, human peripheral blood reticulocytes have upstream RNAs, and these contain the same number of extra 5′ nucleotides as the *in vitro* generated mRNAs. The reticulocyte upstream RNAs are polyadenylylated, and at least their first two exons have been spliced. However, they are probably not translated efficiently, because most of them are not found in polysomes (unpublished observations). Surprisingly, transcription of the upstream RNAs *in vitro* is not inhibited by α-amanitin at 1 μg/ml but is inhibited by 20 μg/ml. This result suggested that the upstream RNAs are transcribed *in vitro* (and probably *in vivo*) by a polymerase similar or identical to polymerase III, which had once been thought to transcribe only 5S, tRNA, and certain viral genes.

Our laboratory is concerned primarily with the function, if any, of the upstream RNAs. Since it might not be feasible to devise appropriate experiments with the human system, we have extended our earlier studies to a more experimentally manipulatable organism, the mouse. Murine fetal, embryonic, and adult tissues are more readily available, and large numbers of immature, colony-forming mouse erythroid cells can be prepared. Furthermore, murine erythroleukemia cell lines can be induced to synthesize adult hemoglobin, making it possible to determine if production of upstream RNA from the globin region is linked to production of mRNA from the same gene.

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The experiments presented here demonstrate that the murine system parallels the human system in several respects, with some differences. The sizes of the mouse upstream RNAs have been determined, and a correlation is demonstrated between dimethyl sulfoxide (Me_2SO)-induced production of globin mRNA and upstream RNAs.

MATERIALS AND METHODS

Cell Culture. Mouse erythroleukemia (MEL) cells (clone 745) were maintained in suspension culture in Ham's F12 medium with 10% fetal bovine serum. For induction, 1% Me_2SO or 0.1 mM hemin was added 3 hr after subculturing at a concentration of 1×10^5 cells per ml. HeLa cells were cultured in Eagle's spinner medium with 10% horse serum.

RNA Purification. Reticulocyte RNA from phenylhydrazine-treated CD-1 mice and total RNA from MEL cells were prepared as described (8–10). Nuclear and cytoplasmic fractions were prepared at 0°C by resuspending pelleted, washed MEL cells in 0.1% Triton X-100/150 mM NaCl/10 mM Tris, pH 7.5/1.5 mM $MgCl_2$. Nuclei were removed by centrifugation at $1000 \times g$ for 10 min. The supernatant (cytoplasm) was adjusted to 0.5% (wt/vol) $NaDodSO_4$. The pellet (nuclei) was resuspended in urea lysis buffer (11), and RNA was purified from both fractions by phenol/chloroform extraction and CsCl centrifugation, as above. In some experiments, RNAs were fractionated by chromatography on oligo(dT)-cellulose (12).

***In Vitro* Transcription in HeLa Extracts.** Extracts were prepared from exponentially growing HeLa cells as described by Manley *et al.* (13). Final dialysis buffer was 100 mM KCl/20 mM Hepes, pH 7.8/10 mM $Mg(OAc)_2$/2 mM dithiothreitol/17% glycerol. Transcription conditions and RNA purification have been described (6). The following circular DNA templates were used: (*i*) template A, mouse DNA from upstream of the β^{major}-globin gene (−354 to −12, *Sau*3A–*Hin*dIII) cloned into *Bam*HI–*Hin*dIII-cleaved pBR322; (*ii*) template B, mouse 5′-flanking and β^{major}-globin DNA (−1400 to +469, *Eco*RI–*Bam*HI) cloned between the *Eco*RI and *Bam*HI sites of pBR322. The +469 site corresponds to a position near the 3′ end of the second coding block. Thus, template B contains 1400 nucleotides of 5′-flanking sequences, the complete first coding block and first intervening sequence, and most of coding block 2; (*iii*) template C: pXb1S, containing a somatic 5S rRNA gene from *Xenopus borealis*.

Blot Analysis of RNA Gels. RNA samples were denatured by heating 5 min at 65°C in electrophoresis buffer [20 mM 3-(*N*-morpholino)propanesulfonic acid (Mops), pH 7.0/5 mM sodium acetate/1 mM EDTA] with 50% deionized formamide/2.2 M formaldehyde. They were then electrophoresed in 1.5% (wt/vol) agarose gels containing 2.2 M formaldehyde

Abbreviations: MEL, mouse erythroleukemia; Me_2SO, dimethyl sulfoxide.

(14, 15) and transferred by blotting for 8 hr in 3.0 M NaCl/0.3 M sodium citrate to Pall Biodyne A nylon (15, 16). The membranes were dried 2 hr in a vacuum oven at 80°C and prehybridized 4 hr at 37°C in 50% formamide/0.6 M NaCl/0.06 M sodium citrate/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin/0.1% $NaDodSO_4$/30 μg of *Escherichia coli* tRNA per ml, pH 6.5. [^{32}P]-RNA probes (10^9 cpm/μg) were prepared by transcription with SP6 RNA polymerase (see below). Blots were hybridized for 18 hr at 37°C in prehybridization buffer that also contained 5×10^5 cpm of RNA probe per ml, 10 mM sodium phosphate at pH 6.5, 50 μg of denatured calf thymus DNA per ml, and 0.33 μg of poly(dT) per ml. Blots were washed once in hybridization buffer at 37°C for 1 hr, twice in 0.45 M NaCl/0.045 M sodium citrate/0.1% $NaDodSO_4$ at 60°C for 30 min, and then twice in 45 mM NaCl/4.5 mM sodium citrate/0.1% $NaDodSO_4$ at 60°C for 30 min. Blots were autoradiographed at −80°C with a single Lightning Plus intensifying screen.

Plasmids for probe synthesis were constructed by inserting a mouse β^{major}-globin −354 to +465 (*Bam*HI–*Hin*dIII) or −354 to −12 (*Sau*3A–*Hin*dIII) DNA fragment downstream from the SP6 promoter in transcription vector pSP65 (Promega Biotec, Madison, WI). [^{32}P]RNAs were synthesized by SP6 polymerase (Promega Biotec) from linearized templates in reactions including 16 μM [α^{32}P]UTP (Amersham 610 Ci/mmol; 1 Ci = 37 GBq). [^{32}P]RNAs were purified in 3% polyacrylamide gels containing 8.3 M urea and eluted from gel fractions as described (17).

S1 Nuclease Analysis of RNAs. Details of end-labeled DNA probe preparation, hybridization, and S1 nuclease digestion have been described (6). The +26 to −494 mouse β^{major}-globin +26 probe was a 520-nucleotide *Hin*cII fragment derived from a 1.8-kilobase *Eco*RI–*Bam*HI subclone of the genomic mouse β^{major}-globin clone MβG2 (18). β^{major}-globin probes +361 and +469 were obtained by cutting the 1.8-kilobase subclone at its unique *Nco* I and *Bam*HI sites, respectively, labeling with polynucleotide kinase, and then digesting with *Eco*RI to release the strand-specific probe. The mouse β^{major}-globin −12 probe was obtained from a subclone of −354 to −12 DNA (*Sau*3A to *Hin*dIII) inserted between the *Hin*dIII and *Bam*HI sites of pBR322. The plasmid was cleaved at the regenerated *Bam*HI (*Sau*3A) site, labeled with polynucleotide kinase, and then digested with *Eco*RI or *Pst* I (which cut in the vector sequence) to release the strand-specific probe. All probes were purified by agarose gel electrophoresis before use. Following S1 nuclease treatment of the hybridization reactions, DNA fragments were electrophoresed in polyacrylamide gels containing 8.3 M urea and autoradiographed.

RESULTS

Upstream Transcripts of the Mouse β^{major}-Globin Gene. Human peripheral blood reticulocytes contain small quantities of RNAs with β-globin mRNA coding sequences and extra nucleotides transcribed from the 5′-flanking region of the gene. There is approximately one upstream RNA per 500 β-globin mRNA molecules, and the upstream RNAs are heterogeneous, with 5′ termini from −235 to −172 (172–235 nucleotides 5′ to the cap site, +1). To determine if analogous transcripts are produced in mouse cells, a DNA probe was 5′-^{32}P-labeled at +26 and annealed to adult reticulocyte RNA. The reaction mix was treated with S1 nuclease, and S1-resistant fragments were electrophoresed. Several bands are observed, corresponding to heterogeneous 5′-flanking segments located 217–107 nucleotides 5′ to the cap site (Fig. 1, lane d). These segments are thus in a similar location, relative to +1, as those of the human β-globin upstream RNAs. Hybridization with the +26 probe demonstrates that the upstream transcripts also contain mRNA sequences, as do the human transcripts. Furthermore, the upstream RNAs bind to oligo(dT)-cellulose (Fig. 1, lane c vs. lane d), suggesting that they are polyadenylylated.

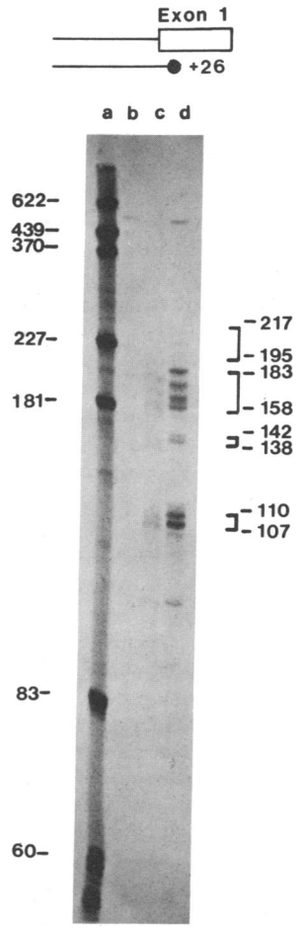

FIG. 1. S1 nuclease protection assay to detect RNAs transcribed from the 5′-flanking region of the mouse β^{major}-globin gene. RNAs were hybridized with a 520-nucleotide β^{major}-globin genomic DNA restriction fragment 5′-^{32}P-end-labeled at the *Hin*cII site (+26). After S1 nuclease digestion the protected fragments were electrophoresed in a 20 × 40 × 0.03 cm 8% polyacrylamide gel containing 8.3 M urea. The gel was autoradiographed with a Cronex Quanta III screen at −80°C for 7 days. Lane a, ^{32}P-labeled *Hae* II fragments of pBR322. Sizes in nucleotides are indicated on the left side. Lane b, 45 μg of *E. coli* tRNA. Lane c, 41 μg of nonpolyadenylylated adult mouse reticulocyte RNA. Lane d, 9 μg of polyadenylylated adult mouse reticulocyte RNA. Positions of the RNA termini relative to the mRNA cap site (+1) are indicated on the right.

A circular template containing mouse β^{major}-globin upstream sequences (−354 to −12) was transcribed in a soluble extract. As determined by S1 nuclease mapping, the *in vitro* synthesized RNAs have the same 5′ termini as the reticulocyte upstream RNAs (Fig. 2*A*, compare lanes c and d). The relative amount of each *in vitro* RNA differed from the corresponding reticulocyte RNA. Longer transcripts with termini upstream of −195 were more prevalent *in vitro*, whereas shorter RNAs with termini between −142 and −107 were more prevalent *in vivo* (Fig. 2*A*, compare lanes c and d). *In vitro* transcription of specific RNAs is inhibited to different extents by 1 μg or 100 μg of α-amanitin per ml (Fig. 2*A*, lanes d–f). Transcription of some, such as that designated −158, is inhibited by α-amanitin at 1 μg/ml (lane e), suggesting that they are synthesized by polymerase II. Transcription of others, such as −183, is reduced at 1 μg/ml but is stimulated at 100 μg/ml (lane f). The phenomenon of increased transcription in the presence of high levels of α-amanitin has been

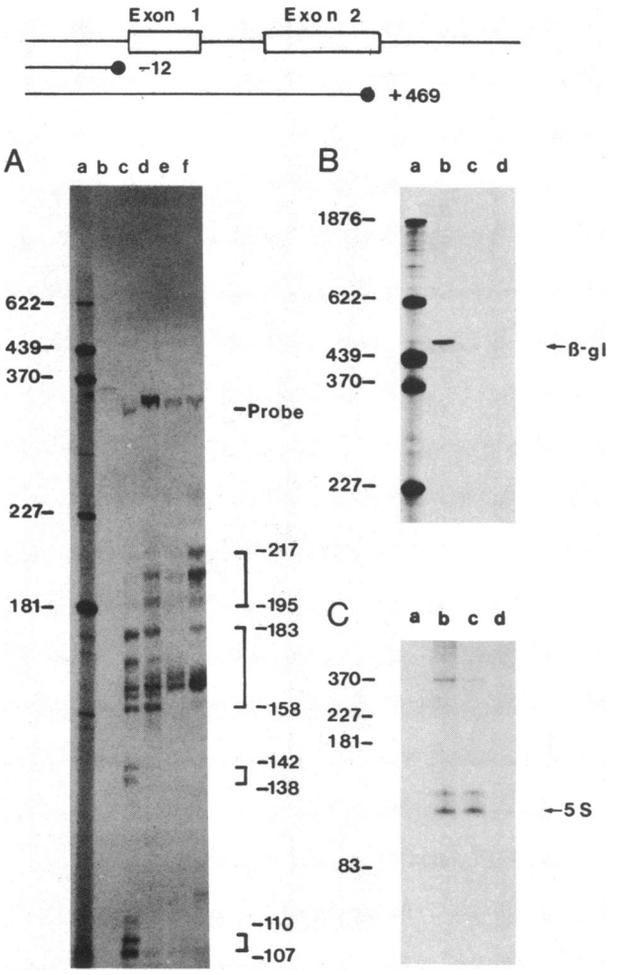

FIG. 2. α-Amanitin sensitivity of mouse β^{major}-globin upstream transcription *in vitro*. RNAs were synthesized *in vitro* by adding different covalently closed, circular DNA templates to HeLa cell extracts. (*A*) DNA template A, a −354 to −12 fragment from the 5′-flanking region of the β^{major}-globin gene. RNAs from 100-μl reactions were hybridized to a DNA fragment 5′-^{32}P-end-labeled at −12 (upper probe, top of figure). After S1 nuclease digestion the protected fragments were electrophoresed. Lane a, ^{32}P-labeled *Hae* II pBR322 DNA markers. Sizes in nucleotides are noted on the left. Lane b, 45 μg of *E. coli* tRNA. Lane c, 50 μg of total adult mouse reticulocyte RNA. Lane d, RNA synthesized *in vitro* without α-amanitin. Lane e, RNA synthesized *in vitro* with α-amanitin at 1 μg/ml. Lane f, RNA synthesized *in vitro* with α-amanitin at 100 μg/ml. Autoradiography was for 14 days with a Quanta III screen. Positions of RNA termini relative to +1 are noted on the right. (*B*) DNA template B, a −1400 to +469 fragment of the β^{major}-globin gene. RNA from 50-μl reactions was hybridized to a DNA fragment 5′-^{32}P-end-labeled at +469 (lower probe, top of figure). Lane a, *Hae* II pBR322 marker; sizes on left. Lane b, RNA synthesized without α-amanitin. Lane c, α-amanitin at 1 μg/ml. Lane d, α-amanitin at 100 μg/ml. Autoradiography was for 24 hr with a Lightning Plus screen. (*C*) DNA template C, pXb1S, which contains an *X. borealis* 5S RNA gene. RNA was synthesized in a reaction containing 40 μM [α-^{32}P]UTP (10 Ci/mmol). After phenol/chloroform extraction, RNA from each reaction was electrophoresed in an 8% polyacrylamide gel, which was then autoradiographed. Lane a, no pXb1S template. Lane b, pXb1S transcripts with α-amanitin at 1 μg/ml. Lane d, pXb1S transcripts with α-amanitin at 100 μg/ml. Autoradiography was for 2.5 hr with a Lightning Plus screen.

described in oocytes and *in vitro* (19, 20). As expected, transcription from the +1 (cap) site is inhibited by >90% with α-amanitin at 1 μg/ml (Fig. 2*B*), whereas transcription from a 5S rRNA gene is inhibited by 100 μg/ml but not by 1 μg/ml (Fig. 2*C*). Templates containing both the upstream and cap site regions generate at least 10 times more +1 than upstream RNA (Fig. 2*B*, lane b). This result has not been quantitated further. To summarize, results with the mouse gene are similar in many, but not all, respects to those with the human gene. Mouse upstream RNAs that include globin mRNA sequences are observed in reticulocytes and are synthesized in cell-free transcription reactions. The reticulocyte and *in vitro* RNAs have the same termini, as determined by S1 nuclease mapping. Some, but not all, of the mouse *in vitro* transcripts are resistant to levels of α-amanitin greater than 1 μg/ml. None of the human upstream RNAs is inhibited by the drug at 1 μg/ml. Some of the mouse transcripts are stimulated by 100 μg/ml (Fig. 2*A*, lane f), and similar results have been observed recently with some of the human transcripts (data not shown). Therefore, it is not clear in either system that a single polymerase is responsible for synthesizing the upstream RNAs. Since transcription of most of the mouse upstream RNAs is resistant to inhibition by α-amanitin at 100 μg/ml (Fig. 2, lane f), these RNAs are not synthesized by RNA polymerase II.

Size of Upstream RNAs. To confirm the existence of upstream RNAs *in vivo* and to determine their size, reticulocyte RNA was electrophoresed, transferred to a nylon support, and hybridized to a [^{32}P]RNA probe specific for the upstream sequences (−350 to −12). A single, diffuse 700- to 900-nucleotide band was observed (Fig. 3 *Left*), primarily in the oligo(dT)-bound fraction (lane B). The upper half of the band appears more intense than the lower half. The size of the band is, at least in part, the result of heterogeneity of the 5′ termini (Fig. 1*A*, lane c). Shorter bands are not detected, although DNA marker fragments of <100 nucleotides were retained on the nylon. This experiment indicates that the upstream RNAs are ≈100–200 nucleotides larger than mature globin mRNA (Fig. 3 *Right*). They cannot contain the complete large intervening sequence. If they did, they would be 600 nucleotides larger than globin mRNA. The data are consistent with the notion that the 3′ portion of the upstream RNAs is similar or identical to globin mRNA, whereas the 5′ portion contains heterogeneous extensions. However, the 3′

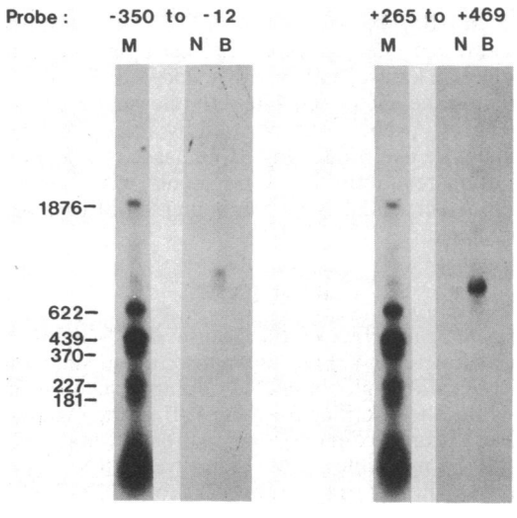

FIG. 3. Analysis of mouse β^{major}-globin upstream RNA by RNA blotting. Two aliquots of adult mouse reticulocyte RNA (20 μg) from the oligo(dT)-bound (lanes B) and nonbound (lanes N) fractions were electrophoresed and blotted. One was hybridized to single-stranded [^{32}P]RNA from the upstream (−12 to −350) region of mouse β^{major}-globin DNA; the other was hybridized to single-stranded [^{32}P]RNA from +265 to +469 in the β^{major}-globin coding sequences. Lanes M contain ^{32}P-labeled *Hae* II fragments of pBR322 of the lengths indicated. Exposure of the marker lanes was 28 hr. Exposure in the *Left* was 24 hr at −80°C with a Lightning Plus intensifying screen. Exposure in the *Right* was 15 min.

structure of these RNAs has not been determined.

Induction of Upstream RNAs in MEL cells. To determine if induction of upstream RNA expression correlates with that of β^{major}-globin mRNA, MEL cells were treated with Me_2SO and collected 24, 48, 72, and 96 hr thereafter. RNA was extracted and analyzed by S1 nuclease protection. No upstream RNAs are detectable in uninduced MEL cells or after 24 hr in Me_2SO (Fig. 4). Upstream RNAs are observed at 48 hr, when the level of globin mRNA significantly increases over background. The levels of both RNAs continue to increase until 72 hr. Upstream RNAs are not observed with the +361 probe (Fig. 4*B*). This is probably because the RNA input is 1% of that in Fig. 4*A* and because we may not be in probe excess in the 72- and 96-hr samples. The additional bands observed in the 48-hr sample (Fig. 4*B*) are not reproducible and do not map 5′ to the +1 cap site. In summary, these data indicate that the kinetics of globin mRNA and globin upstream RNA induction occur more or less in parallel under these conditions.

This result raises the question of whether β^{major}-globin upstream transcripts accumulate under conditions in which only the β^{minor}-globin gene is expressed. Hemin-treated MEL cells continue to divide as they accumulate hemoglobin (21), and β^{major}-globin gene expression is not increased above background (22). Nuclear transcription and DNase sensitivity experiments support the notion that β^{major}-gene transcription is not induced in hemin-treated MEL cells (23, 24). Therefore, hemin "induction" of MEL cells differs from Me_2SO induction. MEL cells were treated with hemin for 72 hr, and the levels of β-globin mRNA and upstream RNAs were measured (Fig. 5). The amount of globin mRNA (presumably β^{minor}) is increased 10-fold over that of the untreated control, but the quantity of upstream RNAs remains below the level of detection. This experiment implies that mRNA production from the β^{minor} gene is not correlated with upstream RNA expression from the β^{major} gene.

DISCUSSION

We have shown that an α-amanitin-resistant RNA polymerase(s) produces RNAs containing both 5′-flanking and structural gene sequences from the mouse β^{major}-globin gene *in vitro*. These transcripts are termed upstream RNAs. Reticulocytes contain RNAs with similar 5′ termini, as determined

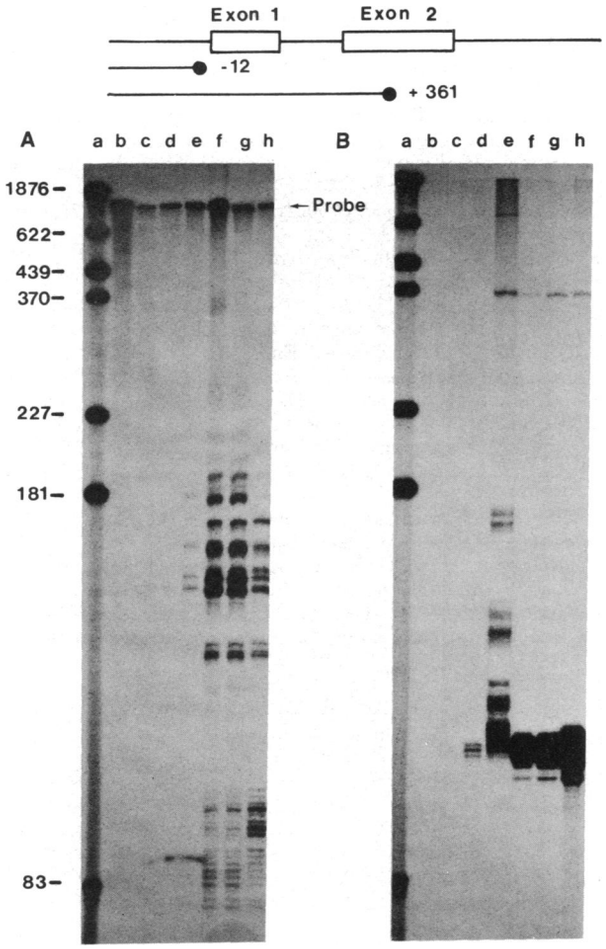

FIG. 4. Induction of mouse β^{major}-globin upstream RNAs in MEL cells treated with Me_2SO. RNAs were hybridized to a DNA fragment 5′-^{32}P-end-labeled at −12 (*A*) or +361 (*B*). The hybrids were digested with S1 nuclease and then electrophoresed in a 30 × 40 × 0.03 cm 8% polyacrylamide gel containing 8.3 M urea for 3.5 hr at 100 W. Lanes a, ^{32}P-labeled *Hae* II fragments of pBR322. Lanes b, 45 μg of *E. coli* tRNA. Lanes h, 14-day mouse fetal liver RNA. The following MEL cell RNAs were analyzed: lanes c, uninduced; lanes d, 24-hr induced; lanes e, 48-hr induced; lanes f, 72-hr induced; lanes g, 96-hr induced. RNA inputs were 50 μg in *A* and 500 ng in *B*. Autoradiography was for 5 days with a Quanta III screen.

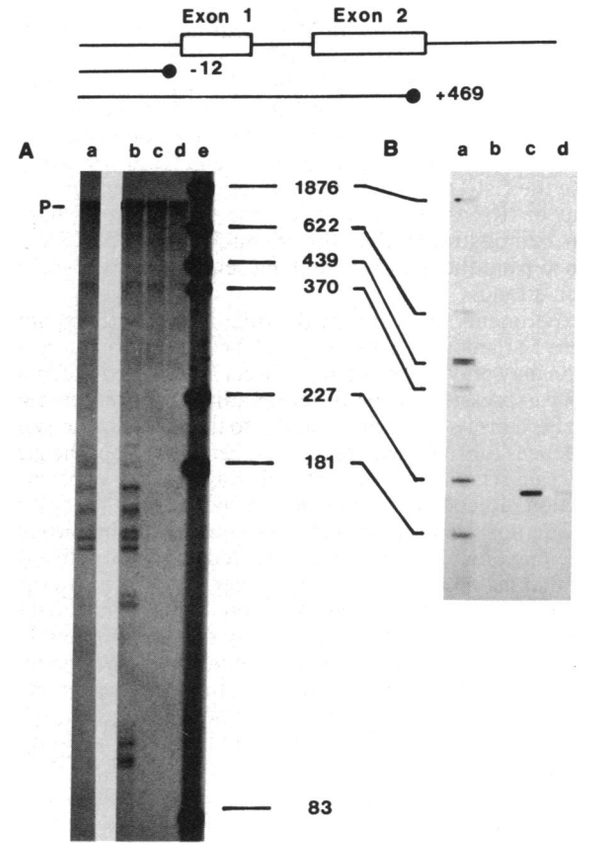

FIG. 5. Assay for β^{major}-globin upstream RNAs in hemin-treated MEL cells. (*A*) RNAs were hybridized to the 5′-^{32}P-end-labeled −12 DNA probe. The hybrids were digested with S1 nuclease and then electrophoresed in an 8% polyacrylamide gel as in Fig. 4. Lane a, 20 μg of RNA from MEL cells induced 48 hr with Me_2SO. Lane b, 20 μg of 14-day mouse fetal liver RNA. Lane c, 20 μg of uninduced MEL cell RNA. Lane d, 20 μg from MEL cells induced 72 hr with hemin. Lane e, ^{32}P-labeled *Hae* II pBR322 markers. Autoradiography was for 3 days with a Quanta III screen. (*B*) RNAs were hybridized to a DNA fragment 5′-^{32}P-end-labeled at +469. After S1 digestion, the hybrids were electrophoresed in a 20 × 20 × 0.07 cm 3% polyacrylamide gel in 8.3 M urea for 3.5 hr at 20 V. Lane a, ^{32}P-labeled *Hae* II pBR322 markers. Lane b, 45 μg of *E. coli* tRNA. Lane c, 3 μg of RNA from MEL cells induced 72 hr with hemin. Lane d, 3 μg of RNA from uninduced MEL cells. Autoradiography was for 1 hr with a Lightning Plus screen.

by S1 nuclease mapping. It thus seems likely that α-amanitin-resistant polymerase(s) transcribes β-globin upstream RNAs in erythroid cells. Upstream RNAs are ≈100–200 nucleotides larger than globin mRNA. They are probably spliced and polyadenylylated in a manner similar to globin mRNA, although the 3′ region of the RNAs has not been characterized. It will be important to know if upstream RNAs from ε-globin (see below) and other genes are also transcribed by an α-amanitin-resistant polymerase(s).

Embryonic cells and some cell lines contain heterogeneous RNAs transcribed from the human embryonic ε-globin gene 5′-flanking region. They are similar to human and mouse β-globin upstream transcripts, their 5′ termini being located primarily within 200 nucleotides of the mRNA cap site (1). Expression of these RNAs in human tissues correlates with ε-globin expression. In contrast, some cell lines express the upstream RNAs but not ε-globin mRNA. We have failed to detect human β-globin upstream RNAs in non-erythroid cell lines, using assays as sensitive as those in the ε-globin experiments (data not shown). Perhaps ectopic expression of upstream embryonic globin RNAs in non-erythroid cell lines ("transformed" cells) is in keeping with the tendency for neoplastic cells to express fetal genes. In any event, our experiments with MEL cells demonstrate a correlation between induction of β^{major} mRNA and upstream RNA expression. A 90-nucleotide RNA prepared from induced MEL cells hybridizes with 5′-flanking sequences of the mouse β^{major}-globin gene (25). We have not observed this RNA with probes that should have detected it, perhaps because the CsCl centrifugation technique used here gives low yields of tRNA-sized RNAs. On the other hand, it has not been demonstrated that the 90-nucleotide RNA is transcribed from the β-globin flanking sequences or, if so, from which strand.

Experiments to determine the role, if any, of transcription of the 5′-flanking region may not be straightforward. This region may play some subtle, indirect role in the induction of globin expression in the differentiating erythroid precursor cell. Perhaps its function is similar to the upstream activating sequences located near some yeast genes (26), but the globin upstream region must be transcribed in order to carry out its function. Recent developments in mouse hematology may provide novel approaches to these questions. In particular, screening of anemic mice has revealed a mutant with a deletion that includes the β^{major}-globin gene (27). If transcription of the upstream region does function in erythroid cell development, a mutation that deletes only this region should generate anemic mice. Perhaps new mutant mice will permit us to test this hypothesis. In addition, the introduction of mutated globin genes into MEL cells should provide a system for testing the potential role of upstream region transcription during Me_2SO induction.

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