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Negative Regulation of the Human ε-Globin Gene by Transcriptional Interference: Role of an Alu Repetitive Element

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The human ε-globin gene has a number of alternative transcription initiation sites which correspond with regions of DNase I hypersensitivity upstream of the canonical cap site. Transcripts originating from the promoters located −4.3/−4.5 and −1.48 kilobase pairs (kbp) and −900 and −200 base pairs (bp) upstream of the major ε-globin cap site can, at certain stages of erythroid differentiation, extend through the gene and are polyadenylated. The 350-bp PolIII transcripts, originating within the Alu repetitive element −2.2 kbp upstream of the cap site, extend in the opposite direction from the gene, are nonpolyadenylated, nucleus confined, and are detectable only in mature K562 cells or mature embryonic red blood cells where the ε-globin major cap site is maximally transcribed. Fragments containing the promoters located between −4.5 and −4.3 kbp upstream of the gene down regulate transcription from the ε-globin gene 20- to 30-fold in a transient expression assay in which both erythroid and nonerythroid cell lines were used. This occurs only when the direction of transcription from the −4.3/−4.5-kbp promoters is towards the gene, and we hypothesize that down regulation is caused by transcriptional interference. Fragments containing the Alu repetitive element −2.2 kbp upstream of the gene can overcome down regulation of the ε-globin gene by the −4.5-kbp element when interposed in the direct orientation between this element and the ε-globin gene.

The human ε-globin gene is expressed during the first 10 weeks of embryonic development. It is then inactivated and remains totally inactive for the remainder of the life span of an individual.

Previous work from our laboratory, summarized in Fig. 1, has shown that the ε-globin gene has multiple sites of transcription initiation located far upstream of the major cap site (1, 2). In the human erythroleukemic cell line K562 (32) or in normal embryonic red blood cells, 10 to 15% of total transcripts originate from the upstream sites. In noninduced K562 cells in which transcripts originate from the major cap site at low levels, the ε-globin upstream promoters give rise to transcripts which extend through the gene and are polyadenylated (see Fig. 1). In fully induced K562 cells, transcripts originating from the far upstream promoters no longer extend through the gene. In addition, the PolIII promoter, located within the *Alu* repeat −2.2 kilobase pairs (kbp) upstream of the gene (14), is transcribed in the opposite direction to the gene and gives rise to 350-base-pair (bp) transcripts, which are nonpolyadenylated and nucleus confined. These transcripts are detectable in vivo, only in fully induced K562 cells or fully differentiated embryonic red blood cells, where the major ε-globin promoter is maximally active (5; Fig. 1). The close association between transcription of this specific *Alu* element and ε-globin transcription has led to the suggestion of a regulatory role (5).

With the exception of the *Alu* promoter, none of the upstream promoters are associated with CAAT or TATA motifs or with any other known regulatory motifs. They are, however, located within regions of DNase I hypersensitivity (7, 33, 37). The correlation of ε-globin promoters and DNase I-hypersensitive sites (DHS) is summarized in Fig. 1. Much evidence supports the view that the alterations of chromatin structure detected by DNase I may locate regions of the genome which play a crucial role in the regulation of gene activity. Direct evidence for protein binding to regulatory DHS has now been provided in a number of systems (15, 16, 23, 35).

Upstream promoters are not unique to the ε-globin gene. They correlate with DHS in the γ-globin and β-globin genes (18) and are located upstream of the ζ- and α-globin genes (21; T. Rutherford, personal communication). They are also found associated with a number of well-documented regulatory elements, including the simian virus 40 (SV40) enhancer (11), the immunoglobulin heavy-chain gene enhancer (27), the thymidine kinase upstream regulatory element (24), the *Xenopus* ribosomal regulatory element (26), and the adenovirus E1A enhancer (28). The correlation of minor promoters with DHS raises the question of whether the transcription process per se has some regulatory significance or is a secondary consequence of accessible chromatin.

In this paper, we describe the regulatory activity of a DHS-promoter located between −4.3 kbp and −4.5 kbp upstream of the ε-globin gene. This element down regulates the gene 20- to 30-fold in erythroid and nonerythroid cell lines, probably by transcriptional interference. Down regulation is prevented by inserting the −2.2-kbp Alu repetitive element between the −4.5-kbp element and the gene. We suggest a similar scenario in vivo, since in relatively undifferentiated K562 cells, transcripts originating at the upstream promoters (including the −4.5 kbp) read through the gene (2; Fig. 1). In these cells, the *Alu* repetitive element is not transcriptionally active. In mature K562 cells, Alu transcripts become detectable (5) and transcripts originating at the −4.5 promoter no longer read through the gene (summarized in Fig. 1). This study confirms a previously stated hypothesis that the ε-globin upstream promoters define regulatory elements and that regulation of gene activity is a direct result of transcriptional activity from these upstream promoters. We also demonstrate, for the first time, a major

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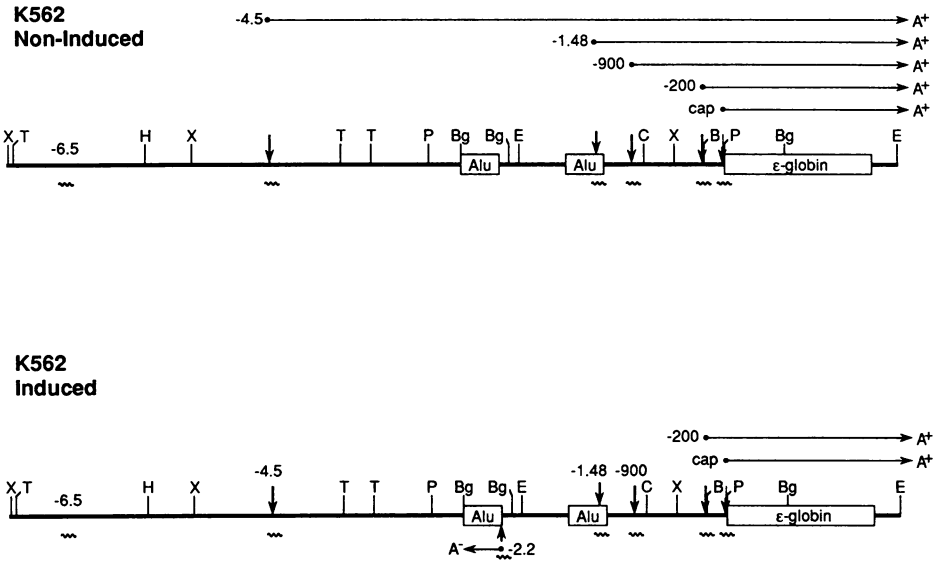

FIG. 1. Summary of DHS-promoters flanking the human ε-globin gene in induced and noninduced K562 cells. The line drawings show the ε-globin gene with 7 kbp of 5′-flanking sequence. DNase I-hypersensitive sites (7, 37) which are active in K562 cells are delineated by wavy lines. Transcription initiation sites (1, 2, 5) are delineated by vertical arrows. The distance from the major cap site is indicated. Arrows above the line drawing indicate transcripts which can extend through the gene and are polyadenylated. The arrow below the line drawing indicates a transcript initiating within the more distal *Alu* repeat, which extends in the opposite direction to the gene, is nonpolyadenylated, and is nucleus confined. Restriction enzyme sites: X, *Xba*I; H, *Hin*dIII; T, *Taq*I; P, *Pvu*II; E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II.

role in gene control for at least one member of the *Alu* family of repetitive elements.

MATERIALS AND METHODS

Plasmids. (i) ε-globin mutants. The plasmid pHRε was constructed by inserting the 3.7-kbp *Eco*RI fragment containing the ε-globin gene into the *Eco*RI site of pAT153. pSVε is identical to pHRε, except that it is derived from the vector pSVOD (25), which contains the SV40 origin of replication as a 311-bp *Eco*RII fragment (4).

The region between the *Xba*I site −7 kbp upstream of the ε-globin cap site and the *Eco*RI site at −2.0 kbp was digested with a variety of enzymes (shown in the line drawing in Fig. 2). Following the addition of *Xba*I linkers, the resulting fragments 2 through 7 were inserted in both orientations into the *Xba*I site located 500 bp upstream of the ε-globin promoter in both pHRε and pSVε. A further construct was made in which the *Taq*I fragment 1 was inserted into the *Cla*I site 800 bp upstream of the ε-globin cap site in the plasmids pHRε and pSVε. The 900-bp *Pvu*II-*Eco*RI fragment containing the more distal Alu repeat was inserted with *Xba*I linkers into the *Xba*I site of pHRε1 and pSVε1.

To allow us to distinguish transcripts derived from ε-globin genes, following transfection into K562 cells from endogenous ε-globin transcripts, plasmids were marked by insertion of an 8-bp nonpalindromic oligomer into the *Pvu*II site 17 bp downstream of the ε-globin cap site.

(ii) Standard plasmid. To provide an internal control for transfection efficiency and losses encountered during RNA preparation and S1 mapping, we cotransfected ε-globin mutants with a standard plasmid, SVβTK. This comprises a herpesvirus thymidine kinase gene driven by the mouse β-globin promoter. Adequate expression of this construct, in a range of cell lines, is ensured by inclusion of an SV40 enhancer. A *Taq*I-*Hin*dIII fragment spanning the β-globin cap site is used as an S1 probe and generates a protected fragment 78 bp in length, mapping to the β-globin cap site.

Cell culture and transfections. Cell lines were grown on SLM medium supplemented with 10% fetal bovine serum (GIBCO). At 24 h prior to transfection, cells were passaged to give 50% confluent cultures, and the medium was replaced 3 to 4 h prior to addition of DNA. Calcium phosphate precipitates were formed as described by Wigler et al. (34). Cells were exposed to precipitates for 14 to 16 h, after which the medium was replaced. Cells were transfected with a range of DNA concentrations (between 0.5 and 40 μg per 75-cm^2 plate) for each construct to determine the point at which maximum expression was obtained.

RNA preparation and analysis. Total RNA was prepared 48 h after transfection by a variation of the method of Chirgwin et al. (9). S1 nuclease analysis of ε-globin 5′ RNA termini was performed by using a single-stranded *Mbo*II fragment spanning the ε-globin major cap site, exactly as described by Allan et al. (2), or by using a 590-bp *Hae*III fragment spanning the −4.3/−4.5-kbp promoter (see Fig. 4).

RESULTS

A far upstream element down regulates transcription of the ε-globin gene. To investigate the hypothesis that distant ε-globin DHS-promoters play a role in regulating activity of the gene, we have digested the region between the *Xba*I site, 7 kbp upstream of the ε-globin cap site, and the *Eco*RI site, 2 kbp upstream of the cap site, with a variety of restriction enzymes (as shown in Fig. 2). The resultant fragments 2 through 7 were cloned by means of *Xba*I linkers, in both orientations, into the *Xba*I site 500 bp upstream of the cap site in the ε-globin plasmids pHRε and pSVε (see Fig. 2). The 3.2-kbp *Taq*I fragment 1 was cloned in both orientations into the *Cla*I site of pHRε and pSVε (see Fig. 2). The plasmids pHRε and pSVε are described in detail in Materials and Methods and by Allan et al. (4). Briefly, pHRε consists of a 3.7-kbp *Eco*RI fragment containing the ε-globin gene inserted into the *Eco*RI site of pAT153. pSVε differs from pHRε only in the possession of an SV40 origin of replication.

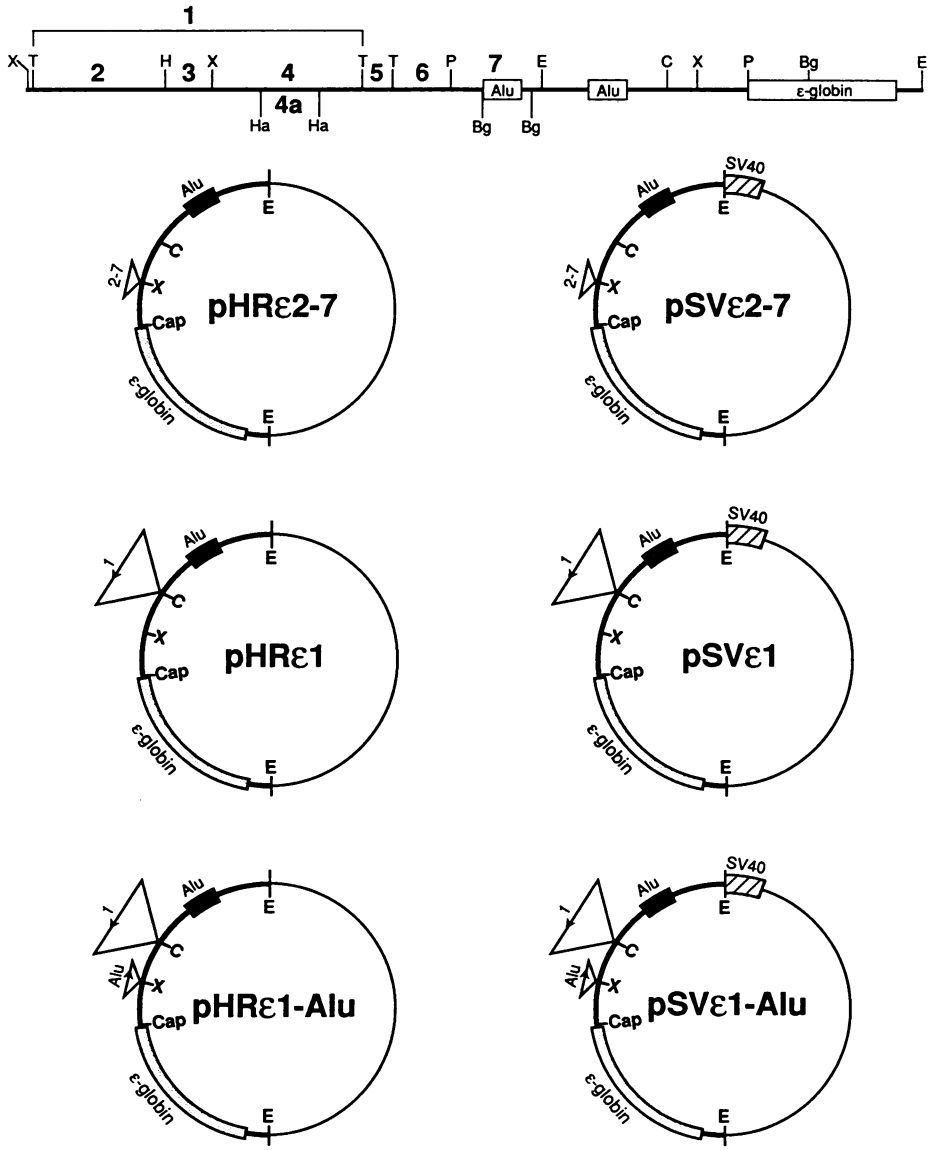

FIG. 2. Construction of ε-globin insertion mutants. The line drawing shows the ε-globin gene with 7 kbp of 5′-flanking sequence. Restriction sites are as follows: C, *Cla*I; X, *Xba*I; H, *Hin*dIII; T, *Taq*I; P, *Pvu*II; E, *Eco*RI; Bg, *Bgl*II; and Ha, *Hae*III. Fragments 1 through 7, generated by appropriate enzyme digestions, are indicated on the line drawing. Fragments 2 through 7 were gel purified and, after addition of *Xba*I linkers, were inserted in both orientations into the *Xba*I site located 500 bp upstream of the major ε-globin cap site in the plasmids pHRε and pSVε. pHRε comprises a 3.7-kbp *Eco*RI fragment containing the ε-globin gene, inserted into the *Eco*RI site of pAT153 (4). pSVε is identical to pHRε, except that it is derived from the vector pSVOD (25) which contains the SV40 origin of replication (4). Fragment 1, which extends from the *Taq*I site (7 kbp upstream of the cap site) to the *Taq*I site (3.8 kbp upstream of the cap site), was inserted directly into the *Cla*I site, which is located 800 bp upstream of the cap site in both pHRε and pSVε. The 900-bp *Pvu*II-*Eco*RI fragment containing the more distal *Alu* repeat was inserted, by means of *Xba*I linkers, into the *Xba*I site of pHRε1 and pSVε1. The direction of transcription of the −4.3-kbp promoter and the *Alu* PolIII promoter are indicated by arrows in the constructs pHRε1-Alu and pSVε1-Alu.

The series of ε-globin plasmids containing different far upstream sequences was transfected into Cos7 and HeLa cells. Increasing quantities of each DNA (from 0.5 to 40 μg/75-cm^2 flask) were transfected in order to determine the concentration at which maximum ε-globin transcription was observed. In Cos7 cells, pSVε reached saturation at 10 μg/75-cm^2 flask of cells, and this concentration was thereafter used for all constructs. RNA was prepared 48 h after transfection and was analyzed by S1 mapping. A single-stranded *Mbo*II fragment spanning the ε-globin cap site was end labeled in the first exon with polynucleotide kinase and [γ-^{32}P]ATP (as shown in the line drawing in Fig. 3). The *Mbo*II probe was hybridized at 57°C to 20 μg of total RNA from each transformed cell line. The hybrids were digested at 37°C for 1.5 h with 1,000 U of S1 nuclease, and digestion products were separated on 6% denaturing polyacrylamide gels. To provide an internal control for transfection efficiency and losses encountered during the S1 mapping procedure, ε-globin mutants were cotransfected with a standard plasmid (SVβTK), comprising a herpesvirus thymidine kinase gene driven by a mouse β-globin promoter (described in Materials and Methods). S1 mapping of RNA derived from

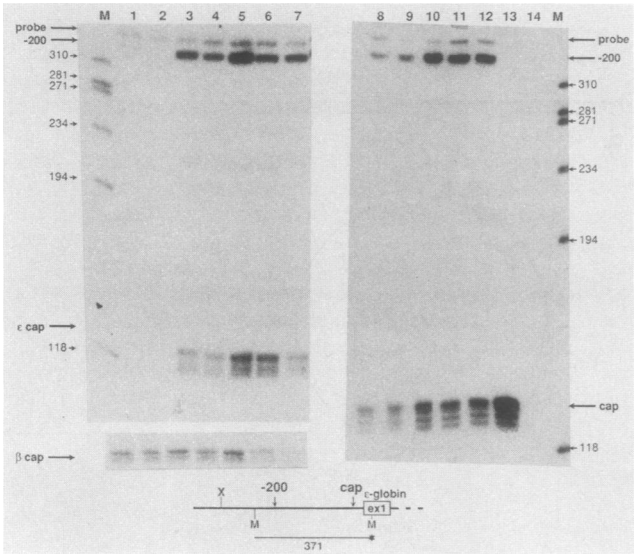

FIG. 3. Transcription of the ε-globin gene after transfection of upstream insertion mutants into Cos7 cells. Cos7 cells were cotransfected with 10 μg of ε-globin mutant and 10 μg of the standard plasmid SVβTK per 75-cm^2 flask (described in Materials and Methods). After 48 h, RNA was prepared and 20 μg was hybridized for 16 h to a single-stranded *Mbo*II fragment flanking the ε-globin cap site (as shown in the line drawing) and to a single-stranded *Hin*dIII-*Taq*I fragment flanking the β-globin promoter. The probes were 5′ end labeled in the first exon of ε-globin (or within the thymidine kinase gene) by T4 polynucleotide kinase and [γ-^{32}P]ATP. Hybrids were treated with 1,000 U of S1 nuclease (Boehringer Mannheim Biochemicals) for 1.5 h at 37°C, and digestion products were denatured and separated on 6% denaturing polyacrylamide gels. Markers (M) are provided by *Hae*III fragments of φX174. The positions of the ε-globin major cap site, the −200 cap site, and the β-globin cap site are indicated on the line drawing and autoradiograph. Autoradiography was for 48 h at −70°C with intensifying screens. The constructs used for transfection were described in the legend to Fig. 2. Lanes: 1, pHRε1; 2, pHRε4; 3, pHRε6; 4, pHRε5; 5, pHRε3; 6, pHRε2; 7, pHRε; 8, pHRε1; 9, pHRε4; 10, pHRε1R; 11, pHRε4R; 12, pHRε; 13, K562; 14, Cos7.

this construct was performed by using a 400-bp end-labeled *Hin*dIII-*Taq*I probe spanning the β-globin promoter. A 78-bp protected fragment maps to the β-globin promoter. Figure 3 shows a typical S1 analysis of the constructs pHRε1-6 after transfection into Cos7 cells. The constructs pHRε1 and pHRε4 show a 20-fold reduction of transcription from the ε-globin −200 cap site and a 4- to 5-fold reduction from the major cap site, compared with the parent plasmid pHRε (compare lanes 1, 2, and 7). Transcription from the β-globin promoter remains constant throughout. None of the other constructs show any difference in transcription compared with pHRε, indicating that the down regulation observed with fragments 1 and 4 does not result from the disruption of potential control sequences after insertion of a foreign DNA into the *Xba*I or *Cla*I sites.

As shown in Fig. 3 (lanes 10 and 11), when fragments 1 and 4 are inserted in the reverse orientation, no difference in ε-globin RNA expression compared with pHRε is observed. Both fragments include a DHS-promoter located between −4.3 and −4.5 kbp upstream of the ε-globin gene described by Allan et al. (2) and Zhu et al. (37) (see Fig. 1), and these fragments exert their down-regulatory effect only when the −4.3/−4.5-kbp promoters are oriented so that transcription is directed towards the ε-globin gene. The down-regulatory element has been further localized to a 590-bp *Hae*III fragment containing the −4.5-kbp DHS-promoter (M. Allan, G. J. Grindlay, P. Bushel, J. Wu, and L. Mendelson, submitted for publication). The results described above have been confirmed by using the pSVε series of constructs and by transfecting both series of constructs into Cos7 and HeLa cells. The transcriptional efficiency of each construct has been obtained from at least five independent experiments with at least two independent preparations of DNA. The extent of down regulation has been accurately quantitated by cotransfection of test plasmids with the standard plasmid SVβTK described above. Gels were scanned by a densitometer, and transcription from the ε-globin −200 promoter and major cap site were compared with transcription from the β-globin promoter. The 20- to 30-fold down regulation of the ε-globin gene by fragments containing the −4.3/−4.5-kbp promoters oriented such that transcription from these promoters is towards the gene has been found under all conditions.

These results raise the intriguing possibility that down regulation is mechanistically correlated with activity of the far upstream promoters and may in fact be achieved by transcriptional interference from the upstream promoter. To confirm that the −4.3- or the −4.5-kbp promoter or both were indeed transcriptionally active in constructs which show down regulation of ε-globin transcription, a 590-bp *Hae*III probe spanning the upstream promoters was gel purified and 5′ end labeled (as shown in the line drawing to Fig. 4). Following transfection of pHRε1 and pHRε4 into Cos7 cells, RNA was analyzed by using this probe. As shown in Fig. 4 (lanes 1 and 2), a 320-bp fragment is protected in both constructs. This corresponds to an initiation site within fragments 1 and 4, normally located −4.3 kbp upstream of the major cap site. This initiation site was originally detected by primer extension analysis in K562 cells and red blood cells from 5- to 7-week-old human embryos (2). In these cell types, the −4.3-kbp promoter is less active than the −4.5-kbp promoter. Apparently, after transfection, promoter usage is altered such that the −4.3-kbp promoter becomes more active than the −4.5-kbp promoter. This phenomenon has been discussed extensively by Allan et al. (3). In any case, we have shown by this experiment that the fragments responsible for down regulating the ε-globin gene possess an active promoter. We will now refer to this as the −4.3-kbp promoter.

We next asked whether fragments containing the −4.3-kbp promoter can down regulate the ε-globin gene in the K562 erythroid cell line. To distinguish transcripts derived from the transfected ε-globin gene from endogenous ε-globin RNA, we constructed the marked plasmids pSVε^m and pSVε4^m, in which an 8-bp nonpalindromic oligomer was inserted into the *Pvu*II site 17 bp downstream of the ε-globin major cap site in the constructs pSVε and pSVε4. These constructs were introduced by calcium phosphate precipitation into K562 cells, and 48 h after transfection, RNA was prepared and hybridized (as described in Materials and Methods) to the marked *Mbo*II-*Mbo*II probe derived from pSVε^m. As shown in Fig. 5, lanes 1 and 2, when an S1 probe derived from the marked gene is hybridized to endogenous ε-globin RNA, the digestion product is truncated at the *Pvu*II site and is therefore 30 bp shorter than the S1 product obtained after hybridization of this probe to RNA derived from the transfected gene and 17 bp shorter than the S1 product generated following hybridization of K562 RNA to the unmarked probe (lane 3). It is clear from Fig. 5 that pSVε^m produces approximately 20- to 30-fold more ε-globin

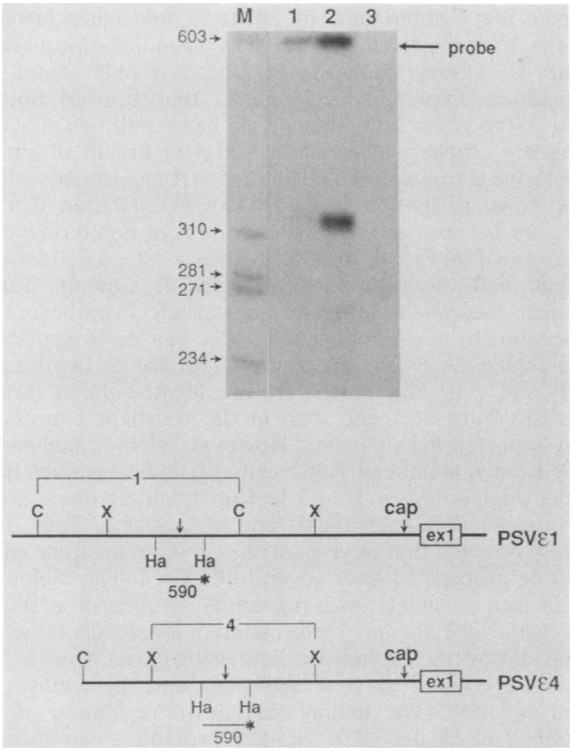

FIG. 4. Activity of the −4.3/−4.5-kbp promoters after transfection of pSVε1 and pSVε4 into Cos7 cells. Cos7 cells were transfected with 20 μg of pSVε1 and pSVε4. Forty-eight hours later, 20 μg of RNA was analyzed by S1 digestion, after hybridization to a single-stranded *Hae*III probe spanning the −4.3/−4.5-kbp promoter region. The probe was labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP. Hybrids were treated with 600 U of S1 nuclease for 1.5 h and separated on 6% denaturing polyacrylamide gel. Markers (M) are *Hae*III fragments of φX174. The line drawings show the composition of transfected plasmids and indicate the probe used for S1 mapping. 1, Fragment 1 inserted into the *Cla*I site of pSVε; 4, fragment 4 inserted into the *Xba*I site of pSVε. Vertical arrows show the major cap site and the position of the −4.3-kbp promoter. Lanes: 1, pHRε1; 2, pHRε4; 3, probe.

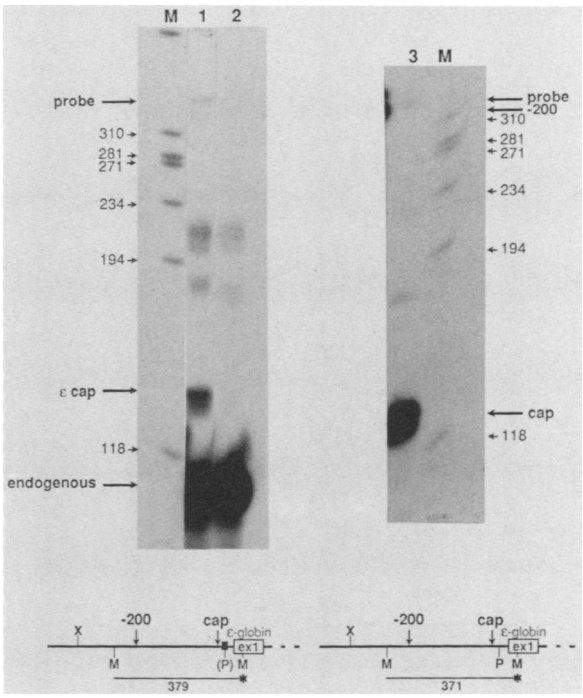

FIG. 5. Down regulation of ε-globin transcription by the −4.3/−4.5-kbp promoter after transfection into K562 cells. K562 cells were transfected with 40 μg/75-cm^2 flask of the constructs pSVε^m and pSVε4^m. Forty-eight hours later, RNA was prepared and 20 μg was analyzed by S1 digestion, after hybridization to a single-stranded *Mbo*II probe derived from pSVε^m. This probe contains a linker inserted into the *Pvu*II site 17 bp downstream of the major cap site (see line drawing) and is therefore 8 bp longer than the unmarked *Mbo*II probe. Hybrids were treated with 1,200 U of S1 nuclease for 1.5 h and separated on 6% denaturing acrylamide gels. The line drawings show marked and unmarked *Mbo*II probes. Positions of the major ε-globin cap site derived from the transfected marked gene and the endogenous cap site are indicated by arrows on the autoradiographs. Lanes: 1, pSVε^m; 2, pSVε4^m; 3, K562 (5 μg) (analyzed with the unmarked 371-bp *Mbo*II probe). Markers (M) are *Hae*III fragments of φX174.

RNA than pSVε4^m does. Thus, fragment 4, when positioned 500 bp upstream of the ε-globin cap site, can down regulate the gene in both erythroid and nonerythroid cell lines.

The *Alu* repeat can overcome the down-regulatory effect on the ε-globin gene of the −4.5-kbp promoter-DHS. The experiments described above have demonstrated that a number of DNA fragments whose common feature is possession of the −4.3/−4.5-kbp ε-globin DHS-promoter can down regulate the ε-globin gene 20- to 30-fold. Down regulation occurs only when the fragment is orientated so that the −4.3/−4.5-kbp promoter transcribes toward the gene (see Fig. 3). It has been shown by S1 analysis that activity of the −4.3/−4.5-kbp promoter is correlated with down regulation. We next asked how the down regulation induced by an upstream element containing the −4.3/−4.5-kbp promoters might be overcome in mature erythroid cells. By using the working hypothesis that down regulation is the result of transcriptional interference, we speculated that the *Alu* repetitive element, which is transcribed specifically in mature erythroid cells by PolIII in the opposite direction from the gene and which is normally interposed between the −4.3/−4.5-kbp promoter and the gene, could be involved in blocking transcriptional readthrough from the upstream promoter.

On this basis, we proceeded to directly test whether down regulation of the ε-globin gene by a fragment containing the −4.3-kbp promoter could be overcome by interposing the *Alu* repeat between the −4.3-kbp promoter and the gene. The constructs used are shown in Fig. 2. pSVε1-Alu comprises the *Taq*I fragment 1 inserted in the direct orientation into the *Cla*I site of pSVε and the 900-bp *Eco*RI-*Pvu*II *Alu* fragment inserted in the direct orientation into the *Xba*I site of pSVε1. The direction of transcription of the PolIII *Alu* promoter and the −4.3-kbp promoter is shown by arrows. Constructs were cotransfected into Cos7 cells with SVβTK. RNAs were prepared 48 h later and analyzed by S1 mapping using the probes described in the legend to Fig. 3. As shown in Fig. 6 (lane 2), pSVε1 transcribes 20- to 30-fold less ε-globin RNA than the parent plasmid pSVε (lane 1) does. However, ε-globin transcription in pSVε1-Alu is equivalent to that of pSVε (lane 3). To test whether a known terminator fragment could overcome the down regulation by fragment 1, the sea urchin H2A histone terminator (30) was inserted into the *Xba*I site of pSVε1 and this construct was also transfected into Cos7 cells. As shown in Fig. 6, lane 6, presence of the terminator fragment was associated with a 20-fold increase in ε-globin transcription compared with pSVε1. To check whether insertion of an arbitrary DNA fragment between fragment 1 and the ε-globin gene would

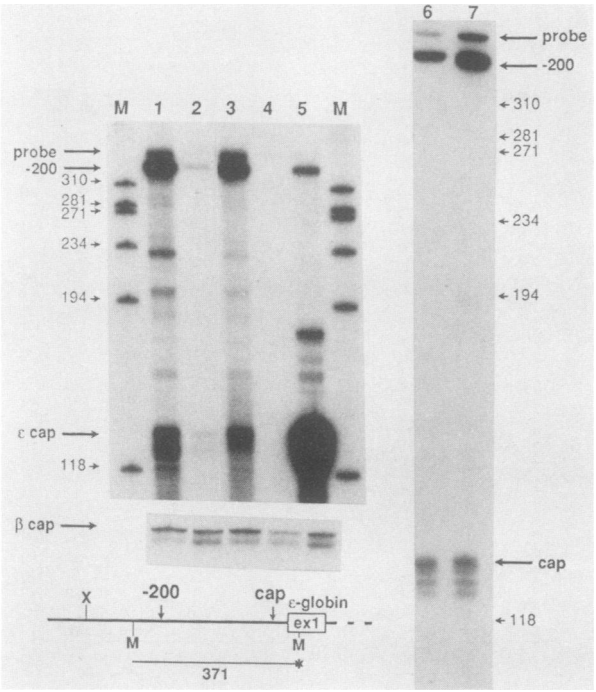

FIG. 6. Alu repeat element overcomes down regulation of ε-globin transcription by fragment 1. pSVε1, pSVε7, and pSVε1-Alu are described in the legend to Fig. 2. pSVε1-5 was made by inserting the 300-bp *Taq*I fragment 5 (Fig. 1 and 2) by means of *Xba*I linkers into the *Xba*I site of pSVε1. pSVε1-T was made by inserting the histone terminator fragment by means of *Xba*I linkers into the *Xba*I site of pSVε1. Constructs were cotransfected into Cos7 cells with the standard construct SVβTK, as described in the legend to Fig. 3. Forty-eight hours after transfection, RNA was prepared and 20 μg was hybridized to a 5′-end-labeled single-stranded *Mbo*II fragment flanking the ε-globin cap site or to a single-stranded *Hin*dIII-*Taq*I fragment flanking the β-globin cap site. Hybrids were treated with 1,000 U of S1 nuclease (Boehringer) for 1.5 h at 37°C, and digestion products were denatured and separated on 6% polyacrylamide gels. Markers (M) are provided by *Hae*III fragments of φX174. Autoradiography was for 48 h at −70°C with intensifying screens. Lanes: 1, pSVε; 2, pSVε1; 3, pSVε1-Alu; 4, pSVε1-5; 5, K562; 6, pSVε1-T; 7, pSVε.

interfere with the down regulation, we inserted fragment 5, which has no associated promoter (see Fig. 2), into the *Xba*I site of pSVε1. As shown in Fig. 6, lane 4, this construct (pSVε1-5) shows equivalent transcription from the ε-globin gene compared with pSVε1. Thus, insertion of the *Alu* repeat element in its natural position and orientation between the −4.3-kbp promoter and the ε-globin gene completely overcomes down regulation of the gene in nonerythroid cells. An identical result is obtained in erythroid cells (not shown). This finding is, again, consistent with the hypothesis that down regulation by fragments containing the −4.3/−4.5-kbp promoter occurs due to transcriptional interference and is supported by the finding that down regulation by fragments containing the −4.3-kbp promoter is overcome by a known terminator.

DISCUSSION

We have shown by using a transient expression assay, in both erythroid and nonerythroid cells, that DNA fragments containing the ε-globin promoter-DHS located between −4.3 and −4.5 kbp upstream of the ε-globin gene (2, 37) down regulate the ε-globin gene by 20- to 30-fold when inserted into the *Xba*I site 500 bp upstream of the major cap site (Fig. 3 and 5). Down regulation is observed only when the upstream promoter is oriented so that transcription is towards the gene, and S1 analysis has confirmed that the promoter normally located −4.3 kbp upstream of the ε-globin gene is transcriptionally active in transformants which show down regulation (Fig. 4). Down regulation can be overcome by insertion of a histone transcription terminator, as described by Proudfoot (30), between the −4.3/−4.5-kbp element and the ε-globin gene (Fig. 6, lane 6). Taken together, these data suggest as a working hypothesis that repression of the ε-globin gene may be caused by transcriptional interference from the upstream promoter. In vivo, the −4.3- and −4.5-kbp promoters are located much further upstream from the gene than in the transient expression assay reported in this paper. However, S1 blot analysis of RNA from noninduced K562 cells (2) indicates that transcripts originating −4.3/−4.5 kbp upstream of the ε-globin gene extended through the 3′ end of the gene. Thus, it is entirely possible that in vivo, such a down-regulatory effect could be propagated over several kilobases. The biological significance of such a down-regulatory mechanism in immature erythroid cells may be to maintain low levels of hemoglobin during the multiple cell divisions which occur between the committed stem cell stage and the mature red blood cell (20). The finding that down regulation of the ε-globin gene by the −4.3/−4.5-kbp promoters can occur in nonerythroid cell lines suggests that *trans* factors required for activity of the upstream promoters are present in all cell types. Support for this comes from our previous report that 5 out of 20 nonerythroid cell lines examined inappropriately express the ε-globin gene. In all cases, transcription occurs exclusively from upstream promoters, while transcripts originating at the major cap site and the −2.2-kbp PolIII promoter are undetectable (2).

Transcriptional interference has been demonstrated in the avian leukosis retrovirus (13). More recently, it has been shown that transcription from an upstream α-globin gene can down regulate expression of a downstream α-globin gene in a transient expression system (30). This down regulation can be overcome by insertion of a histone transcription terminator element between the two genes. Corbin and Maniatis (12) have demonstrated the potential importance of transcriptional interference in developmental gene control of the *Drosophila* alcohol dehydrogenase gene. In vitro studies suggest that transcriptional interference occurs when elongating polymerases from an upstream promoter displace transcription factors from an adjacent promoter and hence impair transcription (6).

It may be significant that the ε-globin silencer described by Cao et al. (8) contains the −200-bp DHS-promoter previously described by us (2, 4, 7). In view of the results presented in the current study, it seems possible that this element may also operate by transcriptional interference.

Down regulation by the −4.3/−4.5-kbp promoter is overcome by an Alu repetitive element. Some mechanism is required in mature erythroid cells to overcome down regulation of the ε-globin gene by the upstream promoter. This could be achieved either by inactivation of the −4.3/−4.5-kbp promoter or by activation of a transcription terminator between −4.5 kbp and the ε-globin gene. The weight of evidence supports the latter hypothesis. Thus, experiments described in Fig. 6 indicate that the *Alu* repetitive element, located −2.2 kbp upstream of the ε-globin gene, completely abolishes down regulation of the ε-globin gene when inter-

posed between the −4.3/−4.5-kbp promoter element and the gene. In addition, down regulation of the ε-globin gene by fragment 1 can be overcome both by the *Alu* repeat and by the histone terminator element described by Proudfoot (30), suggesting that these two elements may be mechanistically similar (Fig. 6). These experiments suggest that the *Alu* repeat may protect the ε-globin gene from the influence of the −4.3/−4.5-kbp promoters in vivo. We suggest that the *Alu* repeat operates to block transcription from an upstream promoter, specifically in mature embryonic erythroid cells, where it is exclusively found to be transcriptionally active (5).

Possible mechanisms by which the *Alu* repetitive element may act to terminate transcribing polymerases are as follows. (i) Transcription of the PolIII promoter from the opposite DNA strand to the upstream promoter may dissociate polymerases originating from the upstream promoter. A similar mechanism is described by Rohrbaugh et al. (31), who find that termination of transcription of the rabbit β-globin gene gradually occurs within a repetitive element located at the 3′ end of the gene. This element is transcribed by PolIII towards the globin gene. (ii) Binding of transcription complexes to the *Alu* PolIII promoter may block the passage of transcribing polymerases. In this model, transcription per se from the *Alu* fragment would be unnecessary and, again, it would be effective irrespective of orientation of the *Alu* repeat. In this context, it is of interest that we have recently shown that the erythroid-specific factor EF1 footprints close to the *Alu* PolIII promoter and that the −2.2-kbp Alu element competes with the ε-globin major promoter for this factor (P. Bushel, J. Wu, and M. Allan, manuscript in preparation). Since the *Alu* effect can also occur in Cos7 cells, it seems unlikely that EF1 is specifically involved, although binding of this factor may be important in derepressing the *Alu* promoter in embryonic erythroid cells. At least three other unidentified factors, which are present in all cell types so far examined, bind within the 380-bp *Bgl*II *Alu* sequence, and one or all of these may be important in blocking the passage of polymerases.

Alu repetitive elements are estimated to occur once every few thousand base pairs throughout the genome (22). They have been reported in the rat growth hormone gene (29) and the mouse α-fetoprotein gene (36) and are interposed between all human α- and β-like globin genes (10, 17). The current study provides the first direct evidence of a function for an *Alu* repeat, and, in light of this, it seems imperative to search for a possible role in gene regulation for other *Alu* elements. *Alu* repetitive elements, transcribed by PolIII, are interspersed between the α1-globin and α2-globin genes and between the ε-, γ-, and β-globin genes. It seems possible that the *Alu* elements may play a role in developmental switching of the globin genes.

The region containing the −2.2- and −4.5-kbp elements has been shown to confer position-independent high-level expression of the β-globin gene in transgenic mice (19). We suggest that the −2.2-kbp Alu element may be involved in this effect, since it could potentially prevent transcriptional interference from adjacent chromosomal genes.

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