Activation of the Human β-Globin Promoter in K562 Cells by DNA Sequences 5′ to the Fetal γ- or Embryonic ζ-Globin Genes

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Abstract

Regulatory sequences of the human fetal γ-globin gene were studied by constructing composite γ/β globin promoters and comparing their function to that of intact β promoters in human K562 cells. The β-globin gene with either 1,600 or 127 basepairs of β promoter sequence was not expressed after stable introduction into K562 cells, consistent with the known inactivity of the β-globin gene in these cells. In contrast, a γ/β promoter composed of a γ fragment spanning positions −408 to −137 joined to the 127-bp β promoter was able to drive the β-globin gene. The gene appeared to be inducible with hemin. A ζ-globin 5′ flanking fragment also activated the β promoter. The function of a series of composite γ/β promoters was then assessed by their ability to drive directly the neomycin resistance gene, again in stably transformed cells. The −408 to −137 γ fragment activated the β promoter in an orientation-specific manner in this assay. Deletion analysis showed that regulatory sequences were present between positions −259 and −137 of the fetal γ-globin gene flanking region.

Introduction

The individual genes of the human α and β globin clusters are temporally regulated so as to bring about the sequential production of embryonic, fetal, and adult hemoglobin during ontogeny. Embryonic hemoglobins are produced early in prenatal life, fetal hemoglobin ($\alpha_2\gamma_2$) during the second and third trimesters, and predominantly adult hemoglobin ($\alpha_2\beta_2$) after birth. The mechanisms controlling these developmental switches are complex and have been studied intensely along diverse lines (1).

Globin genes exhibit three types of specificity. Tissue specificity is reflected by expression only in the erythroid lineage. Developmental specificity is achieved by individual globin genes expressed at various stages leading to characteristic hemoglobin switches. Maturational specificity within the erythroid lineage reflects the increase in the proportion of globin messenger RNA (mRNA) during erythroid cell maturation leading to nearly exclusive globin synthesis in reticuloytes. Globin genes, like other genes, are thought to be regulated by diffusable, *trans*-acting factors that bind to specific *cis*-acting DNA sequence elements within or flanking the genes. Current evidence indicates that tissue, developmental, and maturational specificities of globin gene expression are achieved by the interplay of multiple *cis*-acting elements with several different *trans*-acting factors.

The promoter regions of the globin genes, where initiation of RNA transcription occurs, exhibit tissue specificity in vitro. The human β and γ globin promoter segments, when joined to coding sequences that confer resistance to the aminoglycoside antibiotic G-418 sulfate (G418),[1] were shown to function in erythroid but not in nonerythroid cells (2). By use of human and mouse erythroleukemia cell lines, evidence was also obtained suggesting that the promoter exhibits developmental specificity. These data are consistent with earlier results indicating that the 5′ flanking regions of the globin genes have a characteristic structure in chromatin in normal erythroid cells (3, 4). Furthermore, the sequences immediately upstream from the chicken adult β-globin gene have been shown to bind a developmentally stage-specific factor present in chicken erythrocyte nuclei (5).

Recently, sequence elements with the properties of enhancers have also been identified downstream from globin genes (6, 7) (Bodine, D., and T. Ley, personal communication). In test systems, these sequence elements increase promoter function independent of position and orientation. The element downstream from the human β-globin gene has been implicated in developmental specificity in that it leads to expression of a linked fetal globin gene in adult erythroid cells in transgenic mice (Costantini, F., personal communication).

The property of inducibility of newly introduced globin genes in mouse erythroleukemia cells reproduces the maturational specificity that leads to a high level of globin gene expression in late-stage erythroid cells. The coding sequences of the human β-globin gene appear to confer the property of inducibility when linked either to the γ-globin gene promoter or to a promoter derived from a nonglobin gene (8). However, the β-globin gene promoter when linked to the coding sequences of a noninducible gene also conferred the property of maturational specificity as defined in this assay (8).

Our experiments were designed to define the sequences within the promoter region that confer developmental specificity. We took advantage of the K562 leukemic cell line (9), in which the embryonic (ε, ζ) and fetal ($^{G}\gamma$, $^{A}\gamma$) but not the adult β-globin genes are expressed (10). K562 cells contain three intact copies of an adult β-globin gene. When molecularly cloned and introduced into test cells, the K562 β-globin genes function normally (11, 12). Furthermore, the endogenous β genes can be activated by fusion of K562 cells to mouse erythroleukemia cells (13). The weight of the evidence favors an interpretation that K562 cells contain positive *trans*-acting factors that interact with se-

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1. *Abbreviations used in this paper:* G418, aminoglycoside antibiotic G-418 sulfate; HPFH, hereditary persistence of fetal hemoglobin; Klenow, the large (or Klenow) fragment of the DNA polymerase I enzyme; neo^{R}, neomycin resistance gene, which confers resistance to G418; SV40, Simian virus 40.

quence elements in and around the embryonic and fetal globin genes but lack a comparable *trans*-acting factor required for β-globin gene expression, although this mechanism has not been proven directly.

To define the sequences with which the putative positive *trans*-acting embryonic and fetal factors interact, we constructed composite promoters containing sequences derived from the γ or ζ 5′ flanking region linked to those portions of the β promoter required for constitutive function. Our results indicate that globin gene promoters contain a regulatory element that is an integral part of the promoter in that it functions only in its usual orientation with respect to the coding sequences.

Methods

Plasmid constructions

The plasmid vectors used contained a test promoter driving either the β-globin gene (Fig. 1) or the neomycin gene (14) that confers resistance to G418 (neo^R) (Fig. 2). When the test promoter was a composite globin promoter, the promoter consisted of a γ- or ζ-globin 5′ flanking sequence joined to a 127-basepair (bp) β-globin promoter. The 127-bp β promoter contained the sequences required for correct and efficient transcription of the β-globin gene in other cells (15, 16). Because the β-globin gene is not expressed in K562 cells, however, function of a composite promoter would indicate activation of the promoter and gene by the adjoined γ or ζ fragment. Fig. 3 shows the restriction enzyme sites present within the γ, ζ, and β 5′ flanking regions used in construction of composite promoters.

Plasmids with the β-globin gene. Plasmid vectors carrying the human β-globin gene (Fig. 1) also contained the neo^R gene. In these vectors, the neo^R coding sequences are transcribed from the Simian virus 40 (SV40) early promoter. Vector pCHB614 with the 1.6-kilobase (kb) β promoter had been constructed earlier from a 5-kb Bgl II β-globin fragment (12). Vectors pHL4, pHL7, and pNH1 were derived from a plasmid (pCHB615, described in references 17 and 18) that contained a hybrid γ/β globin gene in which the γ promoter was truncated to position −408.

Vector pHL4 contained the 127-bp minimal β promoter driving the β-globin gene and was constructed using a 0.62-kb Rsa I–Bam HI fragment from the 5′ end of the β gene subcloned between Hinc II and Bam HI of pUC9. The fragment was reexcised as the Hind III–Bam HI piece, and a Cla I linker was attached to the Hind III end after it was blunted by reaction with the large fragment of the DNA polymerase I enzyme (Klenow). The Cla I–Bam HI fragment was used to replace a Cla I–Bam HI fragment in pCHB615, forming a plasmid containing an intact β-globin gene with a unique Cla I site at position −127 (the Rsa I site of the β-globin promoter).

Vector pHL7 contained the −408γ/β promoter and was designed to test for activation of the β-globin gene by γ-globin 5′ flanking sequences. The γ fragment was derived from a 2.4-kb Nco I fragment from pCHB615 (17). It was blunted, and Cla I linkers were attached. A 0.6-kb fragment was obtained by Cla I digestion, which contained pBR322 sequences between Cla I and Bam HI and γ-globin 5′ flanking sequences between the Bam HI–linkered Rsa I site (position −408) and the Nco I site (position −137). This Cla I fragment was inserted into the Cla I site of pHL4. Correct orientation of the insert was verified using the Hind III site located a few basepairs away from the pBR322 Cla I site.

Vector pNH1 contained the ζ/β promoter driving the β-globin gene and was designed to test for activation of the β-globin gene by ζ-globin 5′ flanking sequences. A Cla I–linkered ζ-globin fragment (−390 to −129 Mst II fragment blunted with Klenow) was inserted into pHL4 at its Cla I site.

Plasmids with a globin promoter joined to the Neo^R gene. Plasmids in which a globin promoter was juxtaposed to the neo^R gene are shown in Fig. 2. The neo^R coding sequences had been cloned between the Hind III and Bam HI sites of pBR322. The promoter fragment was placed upstream between the Eco RI and Hind III sites.

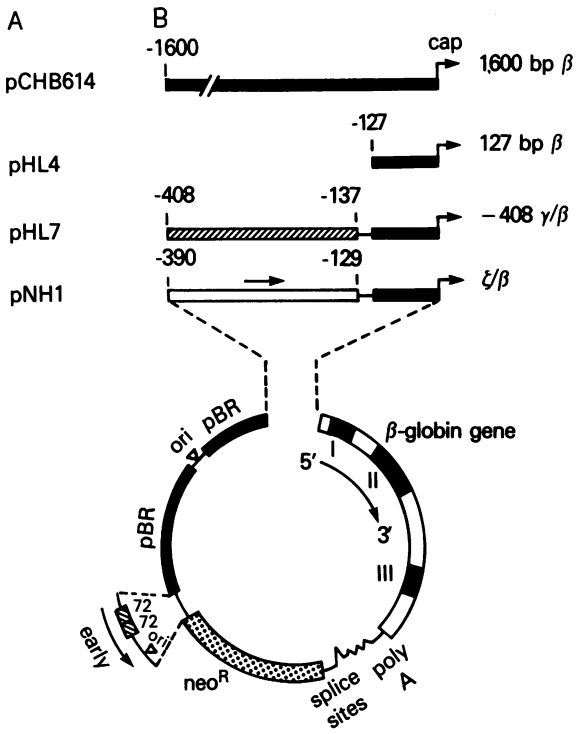

Figure 1. Structure of plasmids carrying the human β-globin gene and the neo^R gene. (*A*) Vector. (*B*) Promoter. The 5′ end of the globin promoter was joined to the pBR322 Bam HI site (for the 1,600-bp β and the −408γ/β promoters) or Cla I site (for all other promoters). The 3′ end of the globin gene was a Bgl II site ligated to Bam HI. The neo^R gene contained the SV40 early promoter (with 72-bp repeated enhancer sequences), splice sites for the t antigen, and polyadenylation signal, and was cloned into the pBR322 Sal I site. The vector also contained an SV40 origin of replication (ori, contained in a 0.31-kb Bst NI fragment) inserted between pBR322 positions 1441 and 2501 (18).

Vector pHL30 contained a 127-bp minimal β promoter sequence identical to that in pHL4, though it was reconstructed from a pUC9 vector carrying a 1.5-kb Bam HI–Nco I β promoter fragment cloned between the Bam HI and Hinc II sites of the polylinker. The Nco I end of the fragment had been blunted to position +49 with S_1 nuclease. A 1.4-kb Bam HI–Bal I β-globin fragment was removed, leaving the β promoter and 5′ untranslated sequences between Bal I and Nco I. A 50-bp Bam HI–linkered Rsa I–Bal I β-globin promoter (Fig. 3) fragment derived from the vector was then ligated in. The promoter was cut out of this vector as an Eco RI–Hind III fragment and inserted into the plasmid containing the neo^R gene.

Vector pHL28 contained the −408γ/β promoter identical to that in pHL7, but it also was reconstructed from the Bam HI–Bal I vector fragment described in the preceding paragraph. The insert was a 0.3-kb γ/β Bam HI–Bal I fragment from pHL7, the Bam HI site being the linker attached to Rsa I at position −408. The −408γ/β promoter was then cut out with Eco RI and Hind III and joined to the neo^R gene.

The −408 revγ/β promoter in vector pHL32b was used to test for enhancer-like function of the γ sequence. The promoter was assembled from a γ-globin fragment spanning positions −408 to −137, obtained as a Cla I–Bam HI fragment from pHL7. The fragment was blunted with Klenow, Eco RI linkers were attached, and the fragment inserted into pHL30. The desired orientation was confirmed via an Apa I site present in the γ-globin 5′ flanking sequence.

In vectors pHL43 and pHL44, the γ fragment from pHL28 was truncated at its 5′ end for the purpose of further localizing the active sequences. In pHL43, the 5′ boundary of the γ fragment was a Bst NI site at position −331. In pHL44, the 5′ boundary was a Hinf I site at

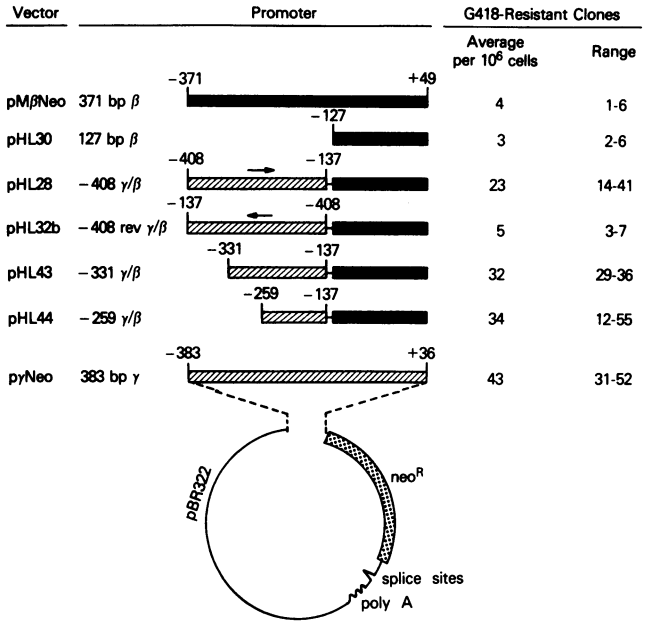

Figure 2. Deletion analysis of the regulatory region 5′ of the fetal γ-globin gene. The globin promoter was cloned between Eco RI and Hind III of pBR322, and the neo^R coding sequence (plus the SV40 t antigen splice sites and polyadenylation signal) was cloned between Hind III and Bam HI. 5 μg of linearized plasmid was used to transfect 10^7 K562 cells by electroporation (19). Viable cells were plated 48 h after shock at a density of 10^4 per 1.5 ml of medium that contained 500 μg · ml^{-1} of active G418. G418-resistant colonies were scored after 11 d of selection. The average number of G418-resistant colonies per 10^6 cells plated under these conditions and the range of frequencies from three or more experiments (except as noted) are tabulated at the right. The total numbers of G418-resistant colonies scored for each promoter were: 371 bp β, 12 colonies; 127 bp β, 21 colonies; −408γ/β, 388 colonies; −408revγ/β, 30 colonies; −331γ/β, 414 colonies (two experiments); −259γ/β, 761 colonies; and 383 bp γ, 451 colonies.

position −259. For the −331γ/β promoter in vector pHL43, a Bst NI γ fragment spanning positions −331 to −157 was blunted with Klenow, Eco RI-linkered, and subcloned into pUC9. The 0.13-kb Eco RI–Apa I fragment from this plasmid then replaced the Eco RI–Apa I fragment of pHL28. Vector pHL44 containing the −259γ/β promoter was constructed by first subcloning a Bam HI fragment from a composite γ/β-globin gene (derived from pCHB603, reference 17) into pUC9. This fragment contained γ sequences extending from a Bam HI–linkered Hinf I site in the 5′ flanking region to the Bam HI site of exon 2. An Apa I–Hind III portion of the subcloned fragment was removed and replaced with the Apa I–Hind III γ/β fragment from pHL28. The γ/β promoter was then cut out as an Eco RI–Hind III fragment and inserted into the neo^R vector.

The vectors containing the 371-bp β promoter (pMβNeo) and the 383-bp γ promoter (pγNeo) were constructed previously (2) and served as controls. The 5′ end of the β promoter was an Acc I site, and the 5′ end of the γ promoter was a Stu I site. All plasmid DNA was prepared by bacterial lysis in Triton X-100 and double banding in cesium chloride gradients.

Transfection of β-globin genes in K562 cells

Vectors containing the globin gene and the neo^R gene with the SV40 promoter (Fig. 1) were linearized with either Aat II or Pvu I and introduced into K562 cells by electroporation. Approximately 5 μg of DNA and 10^7 actively dividing cells were combined in 0.5 ml of ice-cold phosphate-buffered or Hepes-buffered saline in a disposable plastic cuvette fitted with aluminum foil electrodes (19). The cell mixture was pulsed

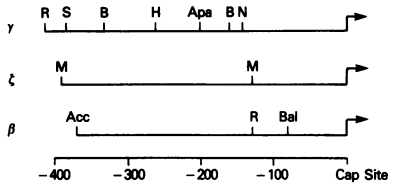

Figure 3. Restriction enzyme cleavage sites of the γ-, ζ-, and β-globin gene 5′ flanking regions used in construction of intact and composite globin promoters. Acc, Acc I; Apa, Apa I; B, Bst NI; Bal, Bal I; H, Hinf I; M, Mst II; N, Nco I; R, Rsa I; S, Stu I. Numbering indicates distances with respect to the cap site.

with a field of 2 kV (from an electrophoresis power supply, model ECPS 2000/300, Pharmacia, Inc., Piscataway, NJ), allowed to stand on ice for 15 min, and then transferred for 24–48 h at 37°C to 10 ml of RPMI 1640 culture medium (Advanced Biotechnologies Inc., Silver Spring, MD) with 10% fetal calf serum. Viable cells were counted with the aid of trypan blue stain and replated at a density of up to 3×10^4 per 1.5 ml in 16-mm plastic wells in medium containing 500 or 1,000 μg · ml^{-1} of active G418 (Gibco, Grand Island, NY). This step represented cloning by limiting dilution as cell growth occurred in only one-third of the wells. DNA from each of 26 of the first-obtained clones was analyzed by Southern blotting and hybridization with a 5′ β-globin probe (20).

RNA analysis

G418-resistant colonies were amplified in selective medium and individually induced with hemin (40 μM, Sigma Chemical Co., St. Louis, MO) for 3–4 d. Approximately equivalent numbers of cells from 9 to 12 individual clones were pooled and RNA prepared by lysis in 6 M guanidine-HCl, 0.2 M potassium acetate, and 2.7% *N*-lauroylsarcosinate (sarkosyl) followed by cesium chloride gradient centrifugation (21, 22). RNA was analyzed by S_1 nuclease mapping with a uniformly labeled probe hybridizing to the 5′ end of the β-globin transcript (23).

G418 resistance assay

Vectors containing a composite γ/β globin promoter joined directly to the neo^R gene (Fig. 2) were linearized with Nde I, and their solution concentrations estimated on an agarose gel before transfection into K562 cells by electroporation as above. Viable cells were plated 48 h after shock in medium containing 500 μg · ml^{-1} G418 at a density of 10^4 per 1.5 ml in 16-mm tissue culture wells. G418-resistant colonies were scored visually at 11 d. Promoter function was measured by the frequency of G418-resistant colonies that formed.

Results

Experimental rationale. We constructed a composite γ/β globin promoter (pHL7, Fig. 1) consisting of a γ fragment spanning positions −408 to −137 joined to a 127-bp β promoter. The β-globin gene driven by this composite promoter, as well as the β-globin gene driven by an analogous ζ/β promoter (pNH1), was expressed in stably transformed K562 clones in preliminary experiments. Because the level of expression of the transfected gene was highly variable among clones, we chose to perform further analyses on pools of clones, as reported below. Thus, a β-globin gene in a vector that also carried the neo^R gene was introduced into K562 cells by electroporation. Representative analyses showed that 77% of the G418-resistant clones had an exogenous β-globin gene at an average copy number of 2.1. Individual G418-resistant colonies were identified, amplified, and induced with hemin. Equal numbers of induced or uninduced cells from 9 to 12 clones were then pooled, and RNA was extracted for assay by S_1 mapping.

To determine the portion of the −408 to −137 γ fragment that contained the putative upstream regulatory sequences, we took advantage of a neo^R assay (2). The assay was more con-

venient than S_1 analysis for assessing function of a series of composite promoters containing portions of the γ 5′ flanking region. The method had been used in this laboratory to assess function of 5′ flanking fragments of the β- and γ-globin genes in several erythroid and nonerythroid cell lines to determine the contribution of promoter and flanking sequences to tissue-specific transcription. Neo^R coding sequences driven by a globin or composite globin promoter were introduced in K562 cells by electroporation, and the cells were selected with G418. The frequency of G418-resistant colonies was taken as an index of promoter function.

Expression of transfected β-globin genes in K562 cells. The β-globin gene containing a 1,600-bp (pCHB614) or 127-bp (pHL4) intact β promoter was not expressed (Fig. 4, lanes *1, 3,* and *4*). In contrast, the β-globin gene driven by the $-408\gamma/\beta$ promoter (pHL7) was expressed and appeared to be inducible with hemin (Fig. 4, lanes *7* and *8*). Two pools of clones were analyzed, and both showed expression and induction of the β-globin gene under control of the composite γ/β promoter. The β-globin gene driven by the ζ/β promoter (pNH1) was also expressed in stably transformed cells. A band was seen, however, for only one of the two pools of clones analyzed (Fig. 4, lanes *11* and *12*). Transcripts from both the γ/β and ζ/β promoters were detected as 132-bp probe-protected fragments, indicating correct initiation at the β-globin cap site.

As internal standards, RNA from the pools was also assayed with a probe for the endogenous γ-globin (Fig. 4, lanes *9* and *10*) or ζ-globin transcripts (lanes *13* and *14*). The induced level of β-globin mRNA was compared by densitometry to the basal amounts of γ-globin or ζ-globin mRNA. We used the basal levels of the endogenous mRNAs for quantitative comparisons, as probe excess was achieved in the S_1 nuclease assays of these samples. The level of β-globin mRNA transcribed from genes with a composite γ/β promoter was 10–30% of the level of basal γ-globin mRNA, and the mRNA transcribed from genes with a composite ζ/β promoter was 30% of the level of basal ζ-globin mRNA. If we assume that uninduced K562 cells produce 250 to 500 molecules per cell of γ- and ζ-globin mRNA (24, 25), then the β-globin transcripts produced from each of the composite promoters was in the range of 25 to 150 copies per cell.

Deletion analysis of the regulatory region 5′ of the γ-globin gene. The 127-bp β promoter, which was not active in driving the intact β-globin gene, gave an average of only three G418-resistant colonies per 10^6 cells plated (pHL30, Fig. 2). A 371-bp β promoter (pMβNeo) gave virtually the same frequency of G418-resistant colonies. A 383-bp intact γ promoter (pγNeo) gave 43 G418-resistant colonies per 10^6 cells. The $-408\gamma/\beta$ promoter (pHL28) also directed expression of the neo^R gene. This promoter gave 23 colonies per 10^6 cells in the neo^R assay. With the orientation of the γ fragment reversed (pHL32b), however, only the background frequency of G418-resistant colonies was obtained. A series of composite γ/β promoters containing varying amounts of the 5′ flanking region were tested. The level of function of the $-259\gamma/\beta$ promoter (pHL44) was comparable to that of the intact γ promoter (pγNeo), despite its lack of γ promoter sequences 3′ of −137. A positive regulatory sequence thus appeared to lie between −259 and −137 of the γ flanking region.

Discussion

A DNA fragment taken from the 5′ flanking region of a γ-globin gene activated the β-globin gene in K562 cells. The stably introduced β-globin gene driven by the composite γ/β promoter was inducible with hemin in these cells. Analysis of a series of

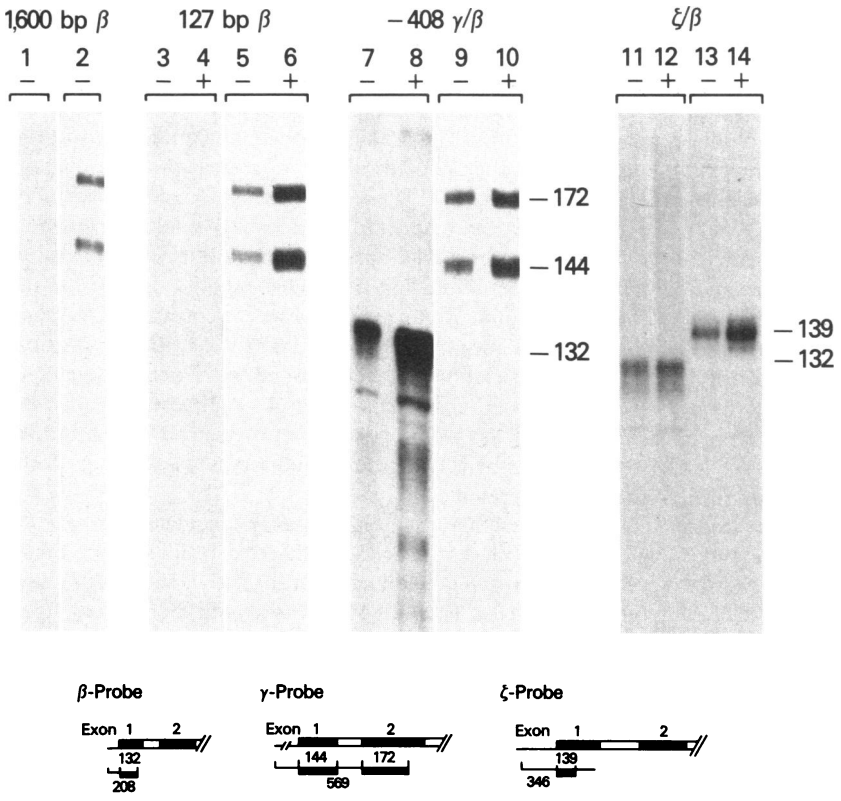

Figure 4. Expression of transfected β-globin genes in K562 cells. K562 cells were transfected with a vector carrying the neo^R gene and a β-globin gene driven by the indicated promoter (refer to Fig. 1). G418-resistant colonies were amplified and cultured for 3–4 d in the presence (+) or absence (−) of 40 μM hemin. Equal numbers of cells from 9 to 12 individual clones were pooled and RNA prepared for S_1 nuclease analysis. 10 μg of RNA was analyzed with a probe to the 5′ end of the β-globin transcript (lanes *1, 3, 4, 7, 8, 11,* and *12*). As internal controls, 2 μg of RNA from the same pools was analyzed with a probe to the γ-globin (lanes *2, 5, 6, 9, 10*) or ζ-globin transcript (lanes *13* and *14*). The probes used are depicted at the bottom of the figure. Protected fragment lengths: β, 132 bp; γ, 144 and 172 bp; ζ, 139 bp.

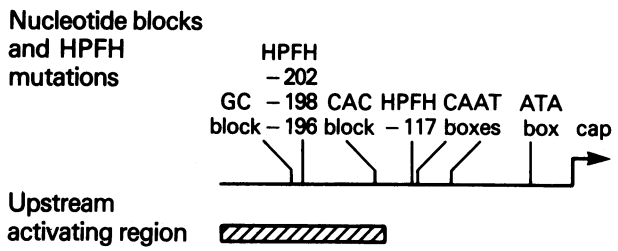

Figure 5. Structural features of the human $^{G}\gamma$- and $^{A}\gamma$-globin gene flanking regions. The GC block is the sequence GGGGGCCCC, and begins at position −208. The single nucleotide mutations at the positions indicated are thought to be causative of HPFH. The CAC, CAAT, and ATA blocks are the conserved sequence elements of the globin promoters. The arrow indicates the mRNA cap site. The upstream activating region is the segment mapped using the promoter constructions shown in Fig. 2.

deletions from the γ fragment showed that the γ regulatory sequences were present between nucleotides −259 and −137. The 5′ flanking region thus appears to be involved in the regulation of the fetal γ-globin gene. The 5′ γ fragment functioned in its native orientation but not when reversed, suggesting that it is a regulatory element of the promoter and not an enhancer. A 262-bp ζ-globin 5′ flanking fragment also activated the β-globin gene in stably transformed cells. Comparison of the human ζ-globin promoter and flanking region to that of the mouse shows that the sequences have been highly conserved, and this homology earlier led to the conclusion that the ζ flanking region has an important function (26).

The relationships between the upstream activating region and the other known features of the flanking region of the human γ-globin gene are summarized in Fig. 5. The region we have defined contains the CAC nucleotide block, which is similar to the constitutive CAC element found in other globin gene promoters (15, 16). The composite γ/β promoters thus have three CAC blocks, compared with two for the β promoter and one for the γ promoter. Because the β-globin promoter with its duplicated CAC block is inactive in K562 cells, triplication of this block is unlikely to account for the function of the γ/β promoters. The γ 5′ activating region coincides with a DNase I hypersensitive region present in the chromatin of fetal liver cells and K562 cells (3, 4). The hypersensitive region has been approximately localized to the 200-bp segment 5′ to the mRNA cap site, and is absent in chromatin of adult bone marrow. Single nucleotide mutations associated with hereditary persistence of fetal hemoglobin (HPFH) also coincide with the putative regulatory region. Mutations occur at positions −196 (27, 28), −198 (29), and −202 (30) of the $^{A}\gamma$ or $^{G}\gamma$ genes, supporting the interpretation that this region has a role in the switch from γ- to β-globin expression. Another HPFH mutation occurs at position −117 (31, 32). The mechanisms by which these mutations lead to HPFH have not been elucidated, but alterations in secondary structure have been proposed to mediate the effect. The −202 mutation, for example, has been shown to abolish a nearby S_1 nuclease hypersensitive site in superhelical plasmids that contain the 5′ flanking sequence (33). The −198 mutation, however, does not appear to cause a structural change detectable by S_1 nuclease (29).

Correct initiation of β-globin mRNAs in our experiments indicated that the γ or ζ 5′ flanking sequences, in combination with the conserved β promoter elements, were sufficient for accurate transcription. The neo^{R} gene driven by a γ promoter has been shown by RNA analysis to be correctly transcribed also (2). The level of expression of the β-globin gene driven by a composite γ/β or ζ/β promoter was ~ 100 mRNA molecules per cell in our pools of clones. These results are comparable to those obtained in similar experiments with a chimeric human γ/rabbit β-globin gene in K562 cells (34). In transgenic mice, low levels of expression of the exogenous gene have also been seen, e.g., a few percent or less of normal (35, 36), although in some cases the levels of transgenic β-globin mRNA have been comparable to the level of endogenous mouse β-globin mRNA (37). Differences in the efficiency of transcription may be due to properties of the integration sites of the transfected genes, phenomena known as chromosome position effects (35).

In the process of analyzing composite globin promoters, we have obtained data on the function of the β-globin promoter and flanking region. The 1,600-, 371-, and 127-bp β promoters were inactive in driving the β-globin gene or the neo^{R} gene in K562 cells. These data are against a simple element lying between positions −1,600 and −127 that acts to inhibit expression of the β-globin gene in these cells (38). Our data are consistent with the recent report of Khazaie et al. (39), in which the human β-globin gene was stably introduced into PUTKO cells, a hybrid line derived from fusing K562 cells with cells of the Burkitt's lymphoma line P3HR-1 (40). The gene was expressed at a low level in 2 out of 20 clones, and transcripts from this gene were truncated at the 5′ end, indicating that the normal β-globin promoter is not active in PUTKO cells. The γ and ζ genes, however, are normally expressed in these cells (41).

The positioning of regulatory sequences upstream of the constitutive elements of the fetal γ-globin gene resembles the general arrangement of control elements for many other genes. Upstream control elements are known for tissue-specific, inducible, or developmentally regulated genes encoding enzymes (42–45), structural proteins (46), polypeptide hormones (47), cellular growth control factors (48), and other products. Multiple regulatory elements should prove to be the rule. For example, two distinct regulatory domains are present upstream of the rat insulin I gene (47). The further upstream domain is an activating region with properties of an enhancer, whereas the other domain confers cell specificity. These neighboring domains presumably act in a cooperative manner to produce the physiologic regulation of the insulin gene. The human α and β interferon genes are also regulated by more than one 5′ element (49–51). The control region of the β-interferon gene consists of a constitutive element juxtaposed to a negative regulatory element (51). Gene induction results in dissociation of a factor bound to the negative element (52). In view of the precise control required for sequential expression of the globin genes, control regions with multiple regulatory elements combining inhibitory and activating effects should be expected.

The finding that 5′ flanking sequences regulate the γ- and ζ-globin genes suggests that the 5′ flanking regions of the other genes in the α- and β-globin clusters are likely to have regulatory function as well. It should now be easier to dissect this potential group of globin regulatory domains by a variety of methods and to construct more complete models of globin gene switching. Determination of the specific nucleotides within the upstream flanking regions that participate in protein binding may contribute to the isolation of the factors important to globin gene expression.

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