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Human Globin Gene Promoter Sequences Are Sufficient for Specific Expression of a Hybrid Gene Transfected into Tissue Culture Cells

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The contribution of the human globin gene promoters to tissue-specific transcription was studied by using globin promoters to transcribe the *neo* (G418 resistance) gene. After transfection into different cell types, *neo* gene expression was assayed by scoring colony formation in the presence of G418. In K562 human erythroleukemia cells, which express fetal and embryonic globin genes but not the adult β-globin gene, the *neo* gene was expressed strongly from a fetal γ- or embryonic ζ-globin gene promoter but only weakly from the β promoter. In murine erythroleukemia cells which express the endogenous mouse β genes, the *neo* gene was strongly expressed from both β and γ promoters. In two nonerythroid cell lines, human HeLa cells and mouse 3T3 fibroblasts, the globin gene promoters did not allow *neo* gene expression. Globin-*neo* genes were integrated in the erythroleukemia cell genomes mostly as a single copy per cell and were transcribed from the appropriate globin gene cap site. We conclude that globin gene promoter sequences extendng from −373 to +48 base pairs (bp) (relative to the cap site) for the β gene, −385 to +34 bp for the γ gene, and −555 to +38 bp for the ζ gene are sufficient for tissue-specific and perhaps developmentally specific transcription.

The human globin genes show tissue-specific and developmentally specific regulation. Thus, these genes are only expressed in differentiating erythroblasts and various genes are preferentially expressed at different stages of ontogeny: ζ and ε in the early embryo; α, $^{G}\gamma$ and $^{A}\gamma$ in the late embryo and in the fetus; and α, δ, and β during adult (postnatal) life (22, 36). The genes are linked in two clusters and lie in the same order 5′ to 3′ as their order of expression during ontogeny, with the α-like globin genes on chromosome 16 (5′-ζ-α2-α1-3′) and the β-like globin genes on chromosome 11 (5′-ε-$^{G}\gamma$-$^{A}\gamma$-δ-β-3′). Based on this organization and on the phenotypes of naturally occurring deletions within the globin gene clusters, it has been argued that gene expression is regulated in *cis* by mechanisms acting over long distances within the clusters (22). However, globin genes with only a few kilobases of flanking sequences have shown regulated expression when transfected into erythroleukemia cell lines (1, 4, 6, 23, 37–39) or when introduced into transgenic mice (3, 25, 35), suggesting that the major *cis* controlling elements are close to or within the genes.

A general strategy for identifying *cis* regulatory elements has been termed reversed genetics. Cloned genes are truncated or mutated in vitro, and their expression is then studied after transfection into cell lines. This strategy has been effective in identifying constitutive *cis* elements in globin gene promoters after transfection into nonerythroid cells (5, 11, 12, 19–21, 27). It has been much less successful in identifying regulatory elements which function specifically in erythroid cells (5, 34), for a number of technical reasons. In particular, it has proved difficult to quantitate differences in gene expression because of variation between individual transfected clones. Furthermore, results with complete globin genes have been equivocal because of the probable presence of multiple regulatory elements within the genes. Thus when β-globin genes with most of the promoter sequences deleted were introduced into murine erythroleukemia (MEL) cells (5, 38) or into transgenic mice (35), the genes showed regulated expression, implying that the promoter sequences are unimportant for regulation. However, this may mean instead that both 5′ and 3′ control elements exist and that either is sufficient for regulated expression (38).

One approach to these problems would be to link isolated potential regulatory elements from the globin genes to an indicator gene such as the chloramphenicol acetyltransferase (CAT) gene (18). We chose, however, to use a selectable marker gene, *neo* (33), which renders cells resistant to killing by the antibiotic G418. Sequences from the globin genes were linked to the *neo* gene, and these hybrid genes were transfected into different cell lines; *neo* gene activity was measured by selecting the cells with G418. In the presence of appropriate regulatory sequences, the *neo* gene should show tissue-specific expression, as if it were a globin gene. Although survival of any cell is an all or nothing phenomenon, the proportion of cells surviving in G418 is a quantitative measure of *neo* gene expression. This follows precisely because integration and expression of a transfected gene varies randomly among individual cells; the proportion of cells which express the gene above the threshold for survival in G418 depends on the relative expression of that gene. In this study we reevaluated globin promoter function by the ability of the promoters to regulate *neo* gene expression. We showed that limited promoter sequences are sufficient for tissue-specific and at least partially developmentally specific gene regulation.

MATERIALS AND METHODS

DNA constructions. Recombinant DNA molecules were constructed by standard methods (10, 26). Briefly, a $^{G}\gamma$ globin promoter stretching from the *Stu*I site at −385 base pairs (bp) (all sites are defined relative to the cap site), blunted and *Bam*HI linkered, to the *Alu*I site at +34 bp (partial *Alu*I digest) was subcloned between the *Bam*HI and *Hin*cII sites of plasmid pUC9. The promoter was cut out of the pUC9 polylinker with *Eco*RI and *Hin*dIII and ligated to a *Hin*dIII-*Bam*HI *neo* gene fragment (33), and the hybrid gene was cloned between the *Eco*RI and *Bam*HI sites of

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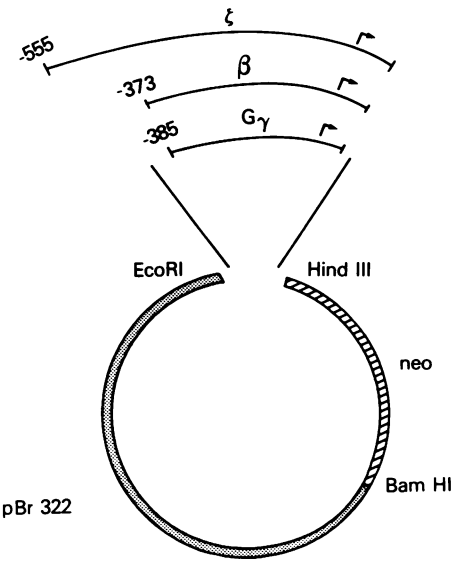

FIG. 1. Structure of globin promoter-*neo* genes.

plasmid pBR322 (Fig. 1). A β-globin promoter fragment from the *Bam*HI site at −1.4 kilobases to the *Nco*I site (blunted with nuclease S1 to +38 bp) was similiarly cloned into pUC9. Sequences from the *Bam*HI site to the *Acc*I site at −385 bp were cut out, the ends were blunted by using Klenow fragment, and the deleted plasmid was recircularized. The deleted promoter was cut out of the polylinker with *Eco*RI and *Hin*dIII, linked to the *neo* gene, and cloned into pBR322 as described above. A ζ-globin promoter fragment from the *Eco*RI site at −555 bp to the *Pvu*II site at +39 bp was cloned into pUC9, recovered as an *Eco*RI-*Hin*dIII fragment, and linked to the *neo* gene, and the hybrid gene was cloned into pBR322 as described above.

Transfection. K562 and MEL C19 TK$^-$ cells were transfected by electroporation (28), using the simplified method of Potter et al. (29). DNA was purified by the Triton X-100 lysis method followed by double banding in cesium chloride density gradients. DNA samples were linearized with *Nde*I, and their concentrations were determined by ethidium bromide staining after agarose gel electrophoresis. Each DNA sample (10 μg) was mixed with 10^7 cells in 0.5 ml of ice-cold phosphate-buffered saline. The mixture was shocked at 2,000 V in an aluminum-foil-lined cuvette as described previously (29). After 15 min on ice, the mixture was transferred to 30 ml of prewarmed tissue culture medium, and the clumps, due to lysed cells, were broken up by gentle shaking. After incubation at 37°C for 48 h, the concentration of viable cells was counted. Cells were cloned at a concentration of 10^4 cells per ml in the presence of 400 μg of G418 per ml (active concentration), either in medium containing 0.8% methylcellulose or by dilution cloning in multiwell dishes. After 14 days of growth, the number of macroscopic colonies was counted by eye.

HeLa cells and mouse 3T3 TK$^-$ fibroblasts (a gift of Dusty Miller) were transfected in different experiments either by electroporation or calcium phosphate precipitation with effectively identical results. Cells were cloned by plating in tissue-culture-treated petri dishes at 10^5 cells per 100-mm dish in the presence of 400 μg of G418 per ml.

Southern blotting. DNA samples were digested with restriction enzymes, analyzed by agarose gel electrophoresis, blotted onto nitrocellulose, and hybridized to nick-translated probes as previously described (30, 32). The probe used was a purified *neo* gene-specific fragment. Slot blotting (Schleicher & Schuell, Inc.) was done in accordance with the protocol of the manufacturer.

RNA analysis. RNA was prepared by the guanidinium thiocyanate-cesium chloride gradient centrifugation method (7). S1 nuclease mapping using M13-derived probes has been described (24). A γ-*neo* probe (see Fig. 3) was made by cloning a *Bal*I (*Eco*RI linkered)-to-*Pvu*II fragment of γ-*neo* into M13mp18, preparation of single-stranded bacteriophage DNA, and in vitro synthesis of a ^{32}P-labeled second strand. An *Eco*RI-*Pvu*I fragment was used for hybridization. The *Pvu*I site was within the flanking vector sequences. A β-*neo* probe was similarly prepared by using an *Eco*RI-to-*Pvu*II fragment of β-*neo* (see Fig. 4).

RESULTS

Gene expression in K562 cells. K562 is a human leukemic cell line which expresses fetal and embryonic but not the adult β-globin genes (30, 31). The globin promoter-*neo* constructs shown in Fig. 1 were transfected into K562 cells, and the cells were selected in G418. After 14 days, the number of macroscopic colonies was scored. To allow for variation in transfection efficiency, the results are expressed as a percentage of the number of colonies obtained with the plasmid pSV2neo (33), in which the *neo* gene is transcribed from the early promoter of simian virus 40. Colony formation is thus a measure of relative promoter strength.

The γ-*neo* promoter was consistently stronger than the strong viral promoter of pSV2neo: 157 ± 30% (five experiments). The ζ promoter was slightly weaker: 83 ± 5% (three experiments). The β promoter however was much weaker: 10.2 ± 3.6% (five experiments). Results from a typical experiment are shown in Table 1 in which gene expression is compared in K562 and HeLa cells. γ-*neo* and ζ-*neo* were strongly expressed in K562 cells, which express the endogenous γ and ζ genes. β-*neo* was weakly expressed in these cells, which do not express their endogenous β-globin genes. All of the globin gene constructs were rarely expressed in the nonerythroid HeLa cells (average, 0.1%; three experiments).

DNA integration and RNA mapping. Individual colonies transfected with γ-*neo* or pSV2neo were expanded in liquid culture. DNA was extracted from the cells, and RNA was extracted before and after induction of hemoglobin synthesis with hemin. A Southern blot of DNA from each of four clones after digestion with *Bam*HI or *Eco*RI and hybridization to a *neo* gene-specific probe is shown in Fig. 2. *Bam*HI cuts out the intact γ promoter-*neo* gene transcription unit of 2.8 kilobases. All four clones showed this band, but clone 2 has an extra deleted or rearranged copy of the gene. *Eco*RI cuts once in the plasmid to yield fragments extending from this site to a site in the flanking cellular DNA, unless there is multiple tandem copy integration, in which case a 6.7-

TABLE 1. Formation of G418-resistant colonies after transfection of globin-*neo* constructs into human erythroid and nonerythroid cell lines

Cell type	Colony formation (% of pSV2neo)			
	pSV2neo	γ-*neo*	β-*neo*	ζ-*neo*
K562 erythroleukemia	100[a]	121	8	84
HeLa	100[b]	0	0	0

[a] A total of 234 colonies; 39 colonies per 10^6 cells per μg of DNA.
[b] A total of 288 colonies; 95 colonies per 10^6 cells per μg of DNA.

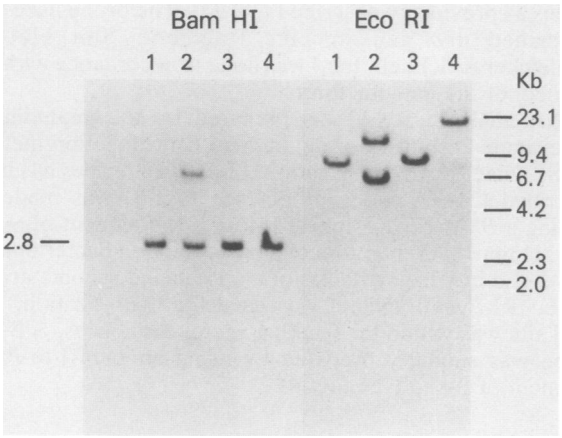

FIG. 2. Southern blot of DNA samples from K562 clones transfected with γ-*neo*. DNA samples from four different clones were digested with *Bam*HI or *Eco*RI, subjected to electrophoresis through a 0.8% agarose gel, blotted onto nitrocellulose, and hybridized to a nick-translated *neo* gene-specific probe. Kb, Kilobases.

kilobase band should be seen. All four clones show different single integration sites, although clone 2 may show tandem integration of 1.5 copies. A much larger series of clones were examined by slot blotting. All had low numbers of, and most probably single, integrated *neo* genes (data not shown).

RNA samples were analyzed by S1 nuclease mapping. Equal amounts of RNA from six equivalent clones were pooled and hybridized to a uniformly labeled M13 probe, shown schematically in Fig. 3. Unhybridized DNA was digested with nuclease S1, and the protected DNA fragments were sized on a denaturing acrylamide gel. The presence of

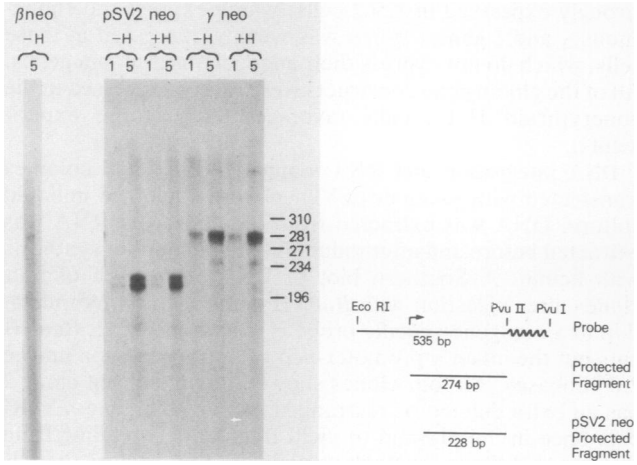

FIG. 3. S1 nuclease mapping of γ-*neo* and pSV2neo transcripts in RNA from K562 clones transfected with the corresponding genes. RNA was extracted from cells grown for 5 days with (+H) or without (−H) 0.04 mM hemin. Pooled RNA from six clones was hybridized to an M13-derived ^{32}P-labeled probe, unhybridized sequences were digested with S1 nuclease, and the protected fragments were sized by electrophoresis on a denaturing polyacrylamide gel. The positions of migration and the size (in base pairs) of marker fragments is indicated to the right of the autoradiograph. The probe and the expected protected bands are shown schematically on the right. Symbols: ↱, γ-globin gene cap site; ~~~, M13-derived sequences. Alternate lanes represent the use of 1 or 5 μg of RNA. The leftmost lane shows transcripts from K562 cells transfected with β-*neo*, mapped with the probe shown in Fig. 4.

TABLE 2. Formation of G418-resistant colonies after transfection of globin-*neo* constructs into mouse erythroid and nonerythroid cell lines

Cell type	Colony formation (% of pSV2neo)			
	pSV2neo	γ-*neo*	β-*neo*	ζ-*neo*
MEL	100[a]	21	34	
3T3 fibroblast	100[b]	0.6	0	0

[a] A total of 44 colonies; 2.5 colonies per 10^6 cells per μg of DNA.
[b] A total of 66 colonies; 33 colonies per 10^6 cells per μg of DNA.

the 274-bp protected fragment indicates that the γ-*neo* transcripts initiated at the correct γ-gene cap site. The pSV2neo protected band is smaller because the transcripts differ from the probe in the 5′ noncoding region. Neither of the transcripts was inducible by hemin.

Gene expression in MEL cells. Friend virus-transformed MEL cells express the mouse adult β-globin genes. Globin-*neo* constructs were transfected into MEL cells in the same way as into K562 cells except that the cells were plated by dilution cloning in multiwell dishes. Results of a typical experiment are shown in Table 2. Both γ- and β-*neo* were expressed in MEL cells, whereas both genes were rarely expressed in mouse 3T3 fibroblasts (average, 0.2%; three experiments). However, the results with MEL cells were much more variable than those with K562 cells. β-*neo* expression varied from 15 to 47% (and was 140% in one experiment). γ-*neo* expression was also variable and sometimes exceeded β-*neo* expression. The transfection efficiency of MEL cells was much lower than that of K562 cells, and therefore results are based on lower total colony numbers (30 to 60 pSV2neo colonies per experiment; five experiments). However, statistical fluctuation does not seem sufficient to explain the degree of variability. We note that the basal uninduced expression of the endogenous globin genes is much lower in MEL cells than in K562 cells and is known to vary spontaneously during growth of the cells in culture. This may explain some of the variability.

RNA mapping of MEL transfectants. Individual MEL colonies transfected with γ-*neo*, β-*neo*, or pSV2neo were grown in liquid culture, and RNA was extracted before or after HMBA induction. Pooled RNA samples were analyzed by S1 nuclease mapping with M13-derived probes (Fig. 4). The protected fragments indicate that both γ- and β-*neo* transcripts were initiated from the correct globin gene cap sites. The β-*neo* transcripts were not inducible by hexamethylenebisacetamido (HMBA), although the endogenous mouse β transcripts were induced 23-fold. Surprisingly, both the pSV2neo and γ-*neo* transcripts were inducible by HMBA, although less strongly than the endogenous mouse β-globin transcripts. All of the colonies were shown by slot blotting to have single or low numbers of integrated *neo* genes.

DISCUSSION

Regulation of gene expression in procaryotes has been elucidated to a considerable extent by genetic experiments. Analogous genetic experiments have not been possible in the study of eucaryotic cell differentiation because the genes of interest cannot in general be used for selection of cells in culture. The long-term aim of our work is to extend the range of genetic experiments possible in mammalian cells. Our strategy is to combine regulatory elements from the human globin genes with selectable marker genes so that the select-

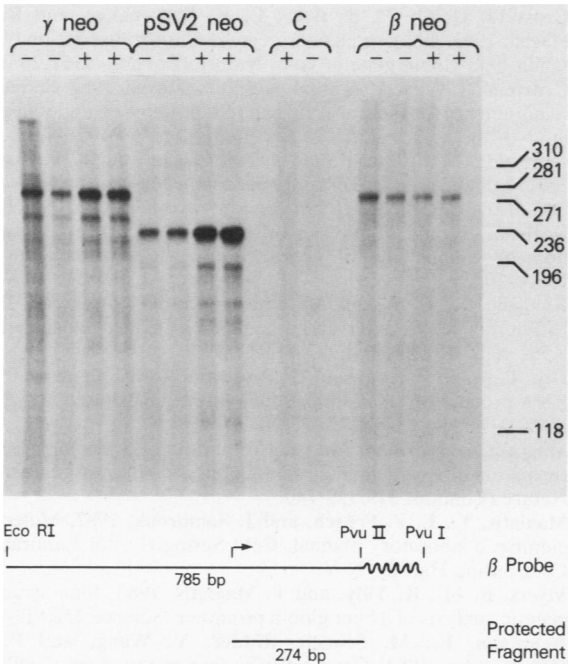

FIG. 4. S1 nuclease mapping of γ-*neo*, pSV2neo, and β-*neo* transcripts in RNA from MEL clones. RNA was extracted from cells grown for 5 days with (+) or without (−) 5 mM HMBA. Pooled RNA from six clones was hybridized to an M13-derived ^{32}P-labeled probe, either the γ-*neo* probe illustrated in Fig. 1 or the β-*neo* probe shown schematically in this figure (bottom), as appropriate. Unhybridized sequences were digested with S1 nuclease, and the protected fragments were sized by electrophoresis on a denaturing polyacrylamide gel. The position of migration and the size (in base pairs) of marker fragments is indicated to the right of the autoradiograph. C, Control (RNA from untransfected MEL cells). Symbols: ↱, β-globin gene cap site; ~~~, M13-derived sequences.

able genes are expressed as if they were globin genes. Such genes could be used to study, and potentially to clone, genes which regulate globin gene expression in *trans*. Similar approaches have been proposed for studying insulin (13) and immunoglobulin gene expression (17).

At the start of this work, only very limited information was available as to the location of *cis* regulatory sequences for mammalian globin genes. Foreign or hybrid globin genes have been transfected into MEL cells, and their response to the induction of hemoglobin synthesis has been determined (4, 6, 37). Transfected β-globin genes with most of the promoter deleted were still inducible, suggesting the presence of a regulatory element within or 3′ to the gene (5, 38). Similar conclusions have come from studies on transgenic mice (35). On the other hand, inducibility of a hybrid β-globin-H2-K gene suggests the existence of a regulatory element between −1,500 and +40 bp in the promoter (38). Equating MEL cell induction with erythroid differentiation is plausible but not certain since the underlying mechanisms are unknown (see below).

Expression of transfected globin genes has also been studied in K562 cells (1, 23, 39), but the results do not identify the sequences responsible for regulated expression. Indirect evidence for the importance of the γ-globin gene promoters comes from the existence of natural point mutations in the promoters which are associated with and may cause nondeletion hereditary persistence of fetal hemoglobin (8, 9, 15, 16).

We chose initially to study globin gene promoter sequences placed in front of the *neo* gene. After transfection into different cell types, expression of these recombinant *neo* genes was measured by the proportion of cells rendered resistant to the antibiotic G418. In K562 cells, promoter fragments from −385 to +34 bp for the γ-globin gene, −373 to +48 bp for the β-globin gene, and −555 to +38 for the ζ-globin gene gave appropriate gene expression: the fetal and embryonic genes were strongly expressed, and the adult gene was weakly expressed. However, in cells expressing β-*neo*, transcription was mainly from the correct cap site (Fig. 3), whereas correct transcription of the endogenous or an intact transfected β-globin gene cannot be detected (1, 23, 39). This may indicate the presence of an additional regulatory element 3′ to +30 bp in the gene. An alternative interpretation of our results is that pSV2neo is expressed 100 times less strongly in K562 cells than in HeLa cells. However, levels of pSV2neo transcripts in K562 and HeLa clones were virtually identical (data not shown).

Both β- and γ-*neo* genes were expressed in MEL cells much more strongly than in nonerythroid cells. Why should a fetal gene be expressed in a mouse adult erythroid cell? In fact, mice do not possess a fetal hemoglobin but express the adult globin genes in the fetal liver. Therefore, they could not be expected to correctly regulate the human fetal gene. Whereas Wright et al. (37) did not observe inducible γ-gene expression in MEL cell transfectants, Anagnou et al. (2) found γ-gene inducibility to be if anything stronger than that of the β gene.

The β-*neo* gene was not inducible during HMBA induction of MEL cells. This may simply mean that the elements responsible for inducibility lie upstream of −380 bp and downstream of +30 bp. It is by no means obvious that the same elements are responsible for the induction response and for tissue specificity. The inducibility of the transcript from the viral promoter in pSV2neo, like the inducibility of a viral transcript observed by Wright et al. (37), does not support such a straightforward view.

Fordis et al. (14) studied the human β- and ε-globin gene promoters by linking them to a CAT gene. Expression of the globin-CAT genes was weak, and the investigators did not show whether transcripts originated from the globin promoters. However, their results were similar to ours in that there was preferential expression of the globin-CAT constructs in erythroid cells and preferential expression of the β-globin-CAT gene in MEL cells compared with that in K562 cells.

Having shown tissue specificity from the globin gene promoters, we are continuing to use this assay system to try to define the minimal promoter elements necessary for tissue-specific transcription of the human globin genes. It should also now be possible to use these constructs to study *trans* regulation of the globin genes by promoter competition, cell fusion studies, and the transfection of *trans*-regulatory DNA.

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