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Genetics

A β-globin gene, inactive in the K562 leukemic cell, functions normally in a heterologous expression system

(erythroid cells/thalassemia/DNA polymorphisms/*trans*-acting regulatory factor/S1 nuclease mapping)

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ABSTRACT **The K562 human leukemia cell is an erythroid-like cell that may serve as a model for the study of globin gene expression in transcriptionally active human erythroid cells. K562 cells express all globin genes with the exception of that for β-globin; failure to produce β-globin could result from an acquired mutation in each of the β-globin genes or from an alteration in the regulatory factor environment of the β-globin gene. To uncover a possible acquired mutation, restriction endonuclease analysis of genomic K562 DNA and expression studies of a cloned K562 β-globin gene were carried out. Restriction endonuclease analysis revealed no structural alteration of the K562 β-globin genes. Analysis of the polymorphic *Ava* II site in intervening sequence 2 of the β-globin gene showed that K562 cells contain two different β-globin alleles, both of which are inactive. A K562 β-globin gene was cloned, ligated into the expression vector pLTN3B, and introduced into COS cells. Transcripts were analyzed by RNA blot, dot blot, S1 nuclease mapping, and primer extension assay. The cloned K562 β-globin gene was transcribed in COS cells as efficiently as a normal β-globin gene introduced into COS cells; the mRNA was 10 S and polyadenylylated; the 5′ and 3′ termini and the processing of transcripts were identical to that of mRNA transcribed from a normal gene. Based on these data we suggest that the absence of β-globin gene expression results not from an alteration in the β-globin gene, but from a quantitative or qualitative alteration in a *trans*-acting factor important in β-globin gene expression.**

Although the structure of the β-like cluster of globin genes has been defined in detail over the last several years (1), an understanding of the regulation of these genes has been elusive. Recent advances in the investigation of globin gene regulation have come, in part, from analyses of naturally occurring disorders of human globin expression. The thalassemias (2) have provided models for examination of sequences important in transcription, splicing, and polyadenylylation. In an analogous manner, investigation of the developmentally aberrant globin expression that can occur in human erythroleukemia cells may provide insights into normal globin gene expression.

The K562 human leukemia cell, although isolated from an adult (3), produces embryonic and fetal hemoglobins, and no adult hemoglobin (4–6). Furthermore, the pattern of transcription from the adult δ- and β-globin genes differs in K562 cells from that normally observed in adult bone marrow. In K562 cells, δ-globin gene transcripts are observed in the absence of β-globin gene transcripts (7); whereas in bone marrow cells δ- and β-globin genes are expressed simultaneously with a 16- to 40-fold excess of β-globin transcripts over those of δ-globin (8). The lack of coincident δ- and β-globin gene expression in K562 cells could result from structural alterations in the K562 β-globin gene(s) or from a change in the environment of diffusible factors. In the present study, we determine if changes in β-globin gene structure are responsible for the lack of β-globin gene expression in K562 cells.

MATERIALS AND METHODS

Cell Culture and Transfection. K562 cells (7) and COS cells (9, 10) were cultured as described. COS cells were transfected using a calcium phosphate DNA coprecipitation technique as described (10).

Analysis of DNA and RNA. Genomic DNA digested with restriction endonucleases was hybridized with the ^{32}P-labeled 1.7-kilobase (kb) *Hha* I fragment of JW102 containing the human β-cDNA (11).

RNA blot analysis was performed on total cellular RNA and on poly(A)$^+$-selected RNA (12). Samples of RNA were denatured for 15 min at 65°C in 10 mM sodium phosphate, pH 7.0/1 mM EDTA/2.2 M formaldehyde/50% formamide; samples were separated electrophoretically in a 1.5% agarose gel (in 10 mM sodium phosphate, pH 7.0/1 mM EDTA/2.2 M formaldehyde) and blotted onto nitrocellulose. After baking for 2 hr at 80°C, the immobilized samples were hybridized (13) against the ^{32}P-labeled 1.7-kb *Hha* I fragment of JW102 containing the human β-cDNA.

RNA was quantitated by a modification of a method of dot blotting (14). S1 nuclease analysis was performed with uniformly labeled probes by a modification (15) of the method of Berk and Sharp (16). RNA was hybridized to double-stranded ^{32}P-labeled probes for 18 hr at 65°C in the presence of 50% formamide. Hybrids were digested with 250 units of S1 nuclease (Miles) and the products were analyzed under denaturing conditions on an 8% polyacrylamide gel. Primer extension analysis was carried out as described (17).

Cloning of the K562 β-Globin Gene. The 6- to 9-kb fragments of a complete *Hin*dIII digestion of genomic K562 cellular DNA were ligated into Charon 21A, packaged *in vitro*, and amplified. Bacteriophage containing the β-globin gene were isolated by the πVX sequence-specific recombination method of Seed (18). The identity of the cloned β-globin gene was verified by hybridization against a ^{32}P-labeled β-globin cDNA and by restriction mapping.

RESULTS

Restriction Enzyme Mapping of Genomic K562 DNA. The structure of the K562 β-globin locus was analyzed by digestion of genomic DNA with *Eco*RI, *Hin*dIII, *Bgl* II, *Pst* I, *Hpa* I, and *Xba* I and by hybridization against a ^{32}P-labeled

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Abbreviations: kb, kilobase(s); SV40, simian virus 40.

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β-globin cDNA (Fig. 1). For each enzyme all hybridizing fragments were of size(s) identical to those found in normal human DNA (19, 20) (Fig. 1, *Lower*). The absence of any mapping abnormality argues against a major insertion, deletion, or rearrangement.

The Presence of Multiple β-Globin Alleles in K562 Cells. β-globin alleles can be distinguished by restriction enzymes with polymorphic sites within the β-globin gene region. A polymorphic *Ava* II site in intervening sequence 2 of the β-globin gene yields, when hybridized against a ^{32}P-labeled β-cDNA probe, either a 2.0- or a 2.2-kb 3′ fragment (21). The 2.6-kb 5′ end of the β-globin gene hybridizes only weakly with the β cDNA in JW102 (22) and was not seen here. K562 DNA contains both the 2.0- and the 2.2-kb fragments (Fig. 2). The faint band below the 2.0-kb fragment represents cross-reaction with the 1.9-kb $^{G}\gamma$- and $^{A}\gamma$-globin fragments (22). K562 cells, heterozygous for the *Ava* II polymorphism, contain two different alleles at the β-globin locus.

Analysis of a Cloned K562 β-Globin Gene. A K562 β-globin gene was cloned using sequence-specific recombination (18). The gene isolated carries the 2.0-kb *Ava* II polymorphic fragment (data not shown). No gene with the 2.2-kb fragment was isolated. The 5.0-kb *Bgl* II fragment containing the K562 β-globin gene (with >1.3 kb of 5′ flanking sequence and >1.5 kb of 3′ flanking sequence) was subcloned into the *Bam*HI site of the expression vector pLTN3B. The vector has been used in the analysis of normal and abnormal globin genes (10, 17). pLTN3B contains the origin of replication and the 72-base-pair repeats of simian virus 40 (SV40) flanked bilaterally by SV40 early mRNA polyadenylylation signals. pLTN3B contains part of the SV40 late region, which is transcribed and can be used as an internal control for transformation efficiency and RNA recovery in analyses that compare the transcription of two β-globin genes. Expression plasmids containing the K562 β-globin gene (pLTN3BKβ) (Fig. 3) or a normal β-globin gene were used to transform COS cells. Total cellular RNA, harvested 48 hr after transfection, was analyzed by RNA blot, dot blot, S1 nuclease analysis, and primer extension.

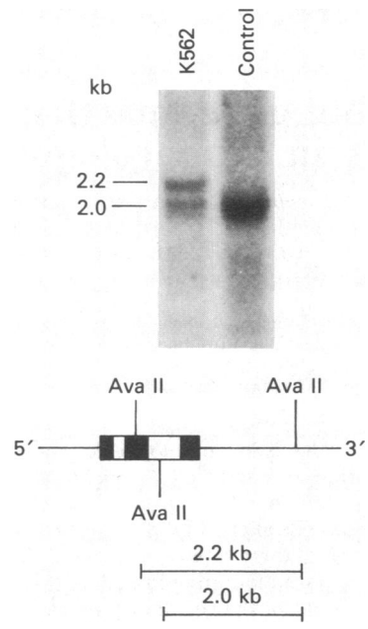

FIG. 2. K562 cell DNA was digested with the restriction enzyme *Ava* II. See legend to Fig. 1.

Total RNA from COS cells transfected with the K562 β-globin gene, total RNA from COS cells transfected with a normal β-globin gene, and human reticulocyte poly(A)$^+$ RNA were compared. Transcripts from the K562 β-globin gene are 10 S, identical in size to transcripts from a normal β-globin gene (Fig. 4*A*). The K562 β-globin gene transcripts are polyadenylylated as demonstrated by the enrichment of mRNA with poly(A)$^+$ selection (Fig. 4*A*).

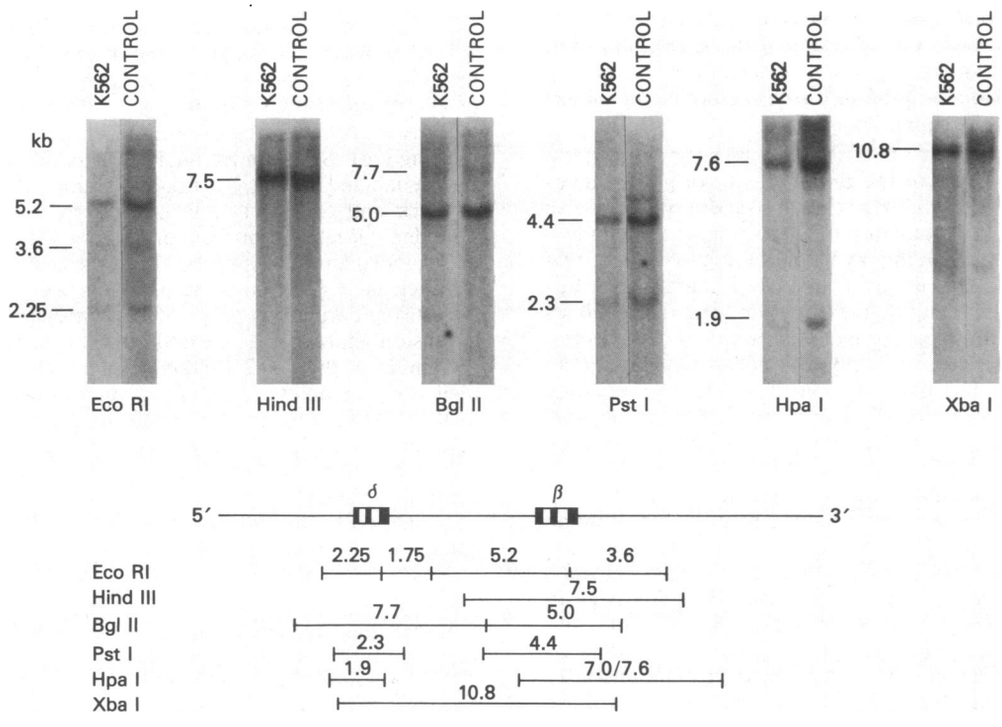

FIG. 1. K562 cell DNA was digested with the restriction enzymes *Eco*RI, *Hin*dIII, *Bgl* II, *Pst* I, *Hpa* I, and *Xba* I; fragments were separated by electrophoresis, blotted onto nitrocellulose, and hybridized against a ^{32}P-labeled β-cDNA probe. Control DNA was obtained from bone marrow cells of a patient homozygous for the β^s allele.

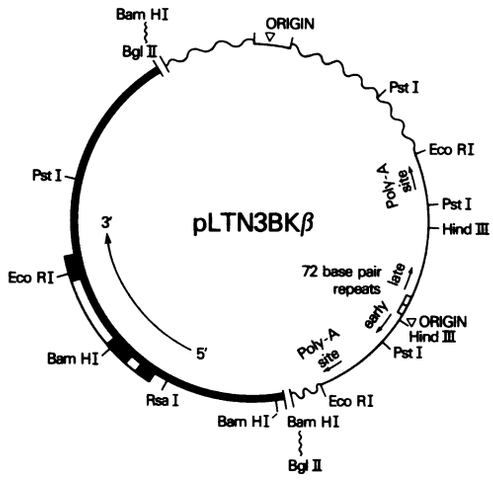

FIG. 3. Diagram of the expression vector pLTN3BKβ. The 5.0-kb *Bgl* II fragment of the K562 β-globin gene was cloned into the *Bam*HI site of pLTN3B in the orientation shown. The heavy solid line depicts K562 sequences; the wavy line depicts pBR322 sequences; and the thin solid line represents SV40 sequences.

By dot blot analysis, the transcriptional efficiency of the K562 β-globin gene in pLTN3BKβ (pLTN3B containing the 5.0-kb *Bgl* II fragment of the K562 β-globin gene) is nearly identical to that of a normal β-globin gene in pLTN3BHβ (pLTN3B containing the 5.0-kb *Bgl* II fragment of a normal β-globin gene) (compare rows a and b in Fig. 4*B*). Transcripts from the SV40 late promoter in pLTN3B were quantitated as an internal control for transformation efficiency and RNA recovery (17). SV40 late transcripts were nearly identical in level in each experiment (compare rows c and d) and suggest that pLTN3BHβ and pLTN3BKβ transform COS cells with an identical efficiency. No SV40 transcripts were identified in up to 7.5 μg of total RNA isolated from COS cells alone (data not shown).

A *Bal* I/*Eco*RI fragment (Probe F) (15) from the normal human β-globin gene was used to map the 5′ end and processing of transcripts from the K562 β-globin gene (Fig. 5, *Left*). K562 RNA protected fragments of 145 and 225 nucleotides, identical in length to fragments protected by RNA from human bone marrow and RNA from COS cells transformed with a vector containing a normal human β-globin gene. In the S1 nuclease mapping figures alone (Fig. 5, *Left* and *Middle*), the K562 β-globin gene lanes cannot be compared quantitatively to the normal human β-globin gene lanes. In these two experiments, the normal human β-globin gene construction contained 1.2-kb less 5′ flanking sequence than the K562 β-globin gene, placing the β-globin promoter of the normal β-globin gene adjacent to the SV40 enhancer. Elimination of the additional flanking sequence is associated with transcriptional enhancement (unpublished observations). The results of S1 nuclease analysis with probe F were confirmed by a primer extension assay (17). An end-labeled *Ava* II/*Bam*HI fragment from the second exon of the human β-globin gene was hybridized with total RNA and extended with reverse transcriptase. RNA from COS cells transformed with the K562 β-globin gene in pLTN3BKβ extended

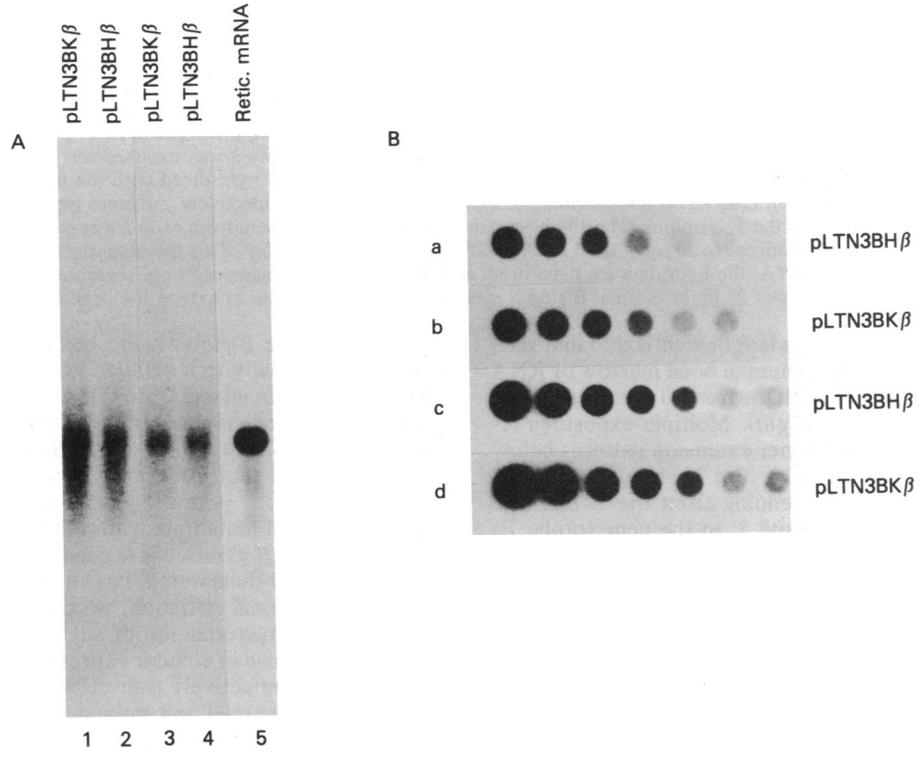

FIG. 4. Size, polyadenylylation, and quantitation of transcripts from the K562 β-globin gene and a normal β-globin gene transfected into COS cells. (*A*) RNA blot analysis. Total RNA was isolated from COS cells transfected with either pLTN3BKβ (the 5.0-kb *Bgl* II fragment of the K562 β-globin gene cloned into the *Bam*HI site of pLTN3B) or pLTN3BHβ (the 5.0-kb *Bgl* II fragment of HβG1, containing the normal β-globin gene, cloned into the *Bam*HI site of pLTN3B). Lanes 1 and 2 contain 20 μg each of total RNA; lanes 3 and 4 contain 2 μg each of poly(A)$^+$ RNA; and lane 5 contains 50 ng of human reticulocyte poly(A)$^+$ RNA as a marker. Lanes 1–4 were exposed for 2 hr and lane 5 was exposed for 1 hr. (*B*) Dot blots. Total RNA was isolated as described in (*A*) and denatured for 15 min at 65°C in 7.5× NaCl/Cit and 5 M formaldehyde (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate). In rows a and b, 12.5, 6.2, 3.1, 1.6, 0.8, and 0.4 μg of RNA was applied; in rows c and d, 25.0, 12.5, 6.2, 3.1, 1.6, 0.8, and 0.4 μg of RNA was applied. Rows a and b were hybridized with the ^{32}P-labeled human β-globin cDNA as described for the RNA analysis; rows c and d were hybridized with the ^{32}P-labeled 1.1-kb *Hin*dIII fragment of pLTN3B containing a portion of the SV40 late region. All blots were exposed to film for 3 hr.

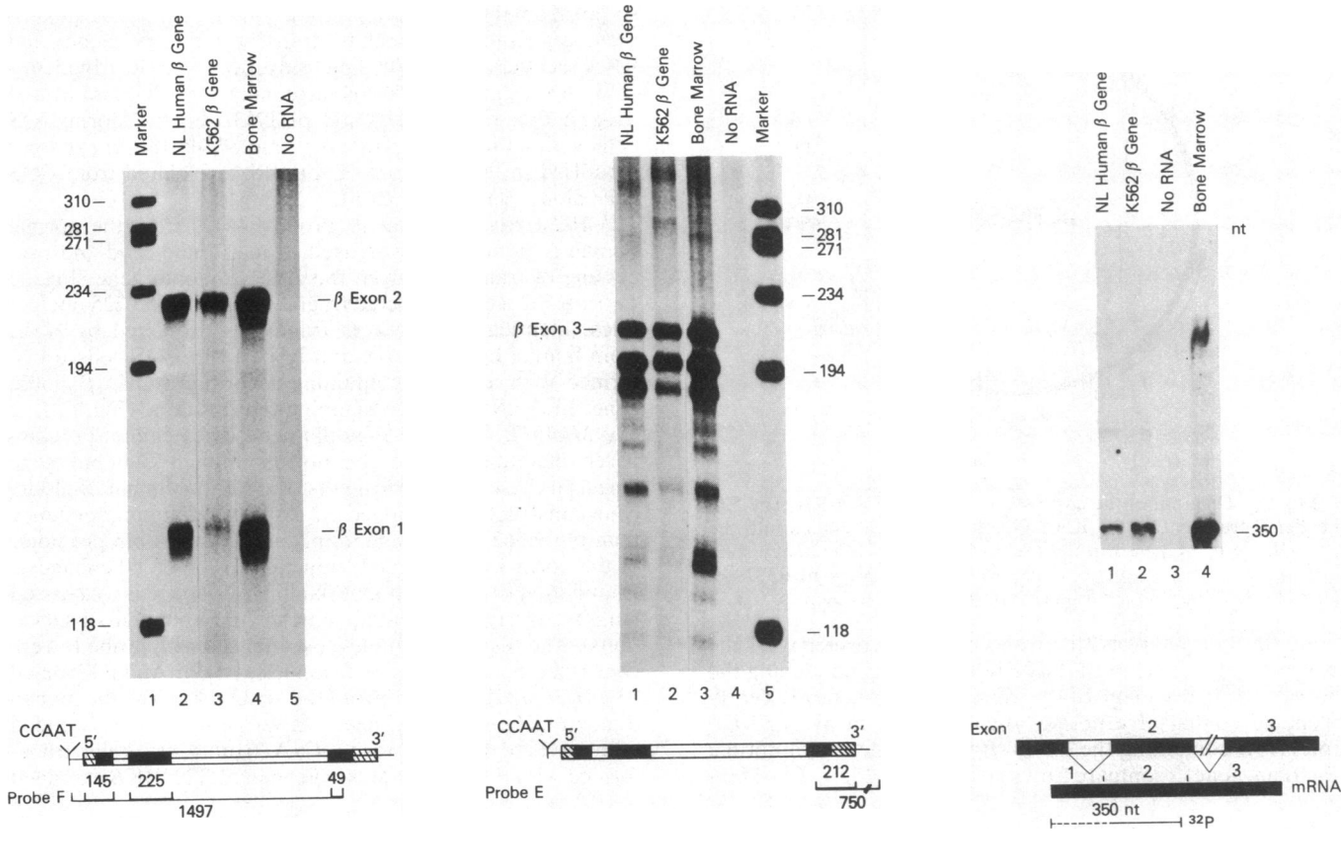

FIG. 5. (*Left*) S1 nuclease analysis of transcripts from the cloned K562 β-globin gene with probe F. Total RNA was hybridized with uniformly labeled probe and digested with S1 nuclease as described. The lower diagram depicts size of fragments protected by normal β-globin mRNA. Fragments were separated on an 8% polyacrylamide sequencing gel. End-labeled *Hae* III-digested φX174 fragments were used as markers. Control RNA was isolated from bone marrow and from COS cells transfected with a normal β-globin gene (the *Rsa* I/*Bgl* II fragment of HβG1 with the *Rsa* I site modified by the attachment of *Bam*HI linkers) subcloned into the *Bam*HI site of pLTN3B. Lanes 2, 3, and 4 contain 9, 10, and 10 μg of RNA, respectively. Lanes 2 and 4 were exposed to film overnight; lane 3 was exposed for 48 hr. (*Middle*) S1 nuclease analysis of transcripts from the cloned K562 β-globin gene with probe E. Total RNA was hybridized with the uniformly labeled probe and digested with S1 nuclease (see above). Lanes 1, 2, and 3 contain 9, 30, and 10 μg of RNA, respectively. All lanes were exposed to film for 48 hr. (*Right*) Primer extension analysis of the 5′ terminus. The 184-base-pair *Ava* II/*Bam*HI fragment from exon 2 was end-labeled at the *Bam*HI site with polynucleotide kinase and hybridized to 20 μg of total RNA in 50% formamide at 55°C for 18 hr. Reverse transcriptase was used to extend the primer to the 5′ end of the mRNA; the hybrids were denatured; and the end-labeled fragments were separated on an 8% polyacrylamide sequencing gel. As shown in the lower diagram, normal β-globin message acts as a template to extend the primer to 350 nucleotides (nt).

the primer to 350 nucleotides—a length identical to that seen in experiments with RNA from human bone marrow or RNA from COS cells transformed with the normal human β-globin gene in pLTN3BHβ (Fig. 5, *Right*). Multiple exposures revealed no differences in the primer extension patterns below 350 nucleotides (data not shown). The 3′ end of transcripts was mapped with a probe extending from the *Eco*RI site within the β gene to the *Pst* I site 3′ to the gene (probe E) (15). The K562 β-globin gene transcripts protected a 212-nucleotide fragment, identical in length to that protected by RNA transcribed from the control vector with a normal β-globin gene and RNA from human bone marrow (Fig. 5, *Middle*). The additional bands below 212 nucleotides presumably represent S1 nuclease "nibbling" of the A+T rich region in exon 3 (15).

DISCUSSION

β-Globin gene expression disappears with a variety of *cis*-acting mutations (2), any one of which might be found in K562 cells. Most obviously, deletions of the β-globin gene can abolish β-globin expression (23). However, at the level of restriction endonuclease mapping, we show that K562 cells contain at least two intact β-globin genes. Deletions adjacent to the β-globin gene can affect β-globin gene expression. In one such deletion, loss of the $^{G}\gamma$-, $^{A}\gamma$-, and δ-globin genes produces γδβ-thalassemia. Although in this form of thalassemia the β-globin gene has a normal sequence and functions normally in a cellular expression assay, it cannot be transcribed *in vivo* (24). In this case, the loss of >40 kb of adjacent sequence is associated with the absence of β-globin gene expression. Mapping of the β-like cluster (ref. 6; see above) showed that K562 cells contain no similar deletion and express both γ- and δ-globin genes (4–7). Apart from deletions, small mutations within the β-globin gene can dramatically alter β-globin expression. Analysis of genes from patients with β-thalassemia has revealed mutations that affect transcriptional initiation, processing, and polyadenylylation (2). Characterization of such gene defects has relied heavily on the use of cellular expression systems (2) in which globin genes are actively transcribed and the transcripts are accurately processed and polyadenylylated. Mutant globin genes are identified by a decrease in the level or disappearance of correctly initiated, processed, and polyadenylylated transcripts. We cloned a K562 β-globin gene for analysis in a heterologous expression system. Transcripts from the cloned K562 β-globin gene are identical in size, processing, polyadenylylation, and quantity to those from a normal β-globin gene.

Although the cloned K562 β-globin gene functions like a normal β-globin gene in a heterologous expression system, it is conceivable that mutation in a *cis*-acting sequence located outside the cloned region could be responsible for the lack of

β-globin gene expression within K562 cells. To produce the observed phenotype (i.e., absence of β-globin transcripts), such mutations, recessive in nature, must strike each β-globin gene in K562 cells. Karyotypic analysis of our K562 cells shows partial triploidy and three copies of the chromosome that bears the β-globin locus, chromosome 11 (unpublished data). Furthermore, our analyses of the *Ava* II polymorphic site in intervening sequence 2 of β-globin distinguishes two alleles at the β-globin locus in K562 cells. Therefore, a mutation outside the cloned region must involve at least two, and possibly three, β-globin alleles. Such an occurrence seems unlikely. Further confirmation comes from work in our laboratory that demonstrates that a K562 β-globin gene can be expressed within the K562 cell. K562 cells, cultured for 4 days in the presence of 5-azacytidine, contain transcripts for β-globin (25). Therefore, at least one K562 β-globin gene can function in the native cell. Our findings suggest that the lack of β-globin expression in K562 cells results from an alteration in the environment of diffusible factors and not from mutation in each of the K562 β-globin genes. Support for this conclusion also comes from preliminary results on the transient expression of a cloned K562 β-globin gene by others (26). Furthermore, when K562 cells were stably transformed with a cloned normal β-globin gene, none of three lines produced β-globin transcripts (26). The effect of chromatin structure on these results remains to be defined.

Although *trans*-acting factors and their role in gene expression have been well characterized in prokaryotic systems, in eukaryotic systems the role of such factors is less well understood. In *Xenopus*, a purified 40,000-dalton protein has been implicated in maintenance of the differentiated state of 5S rRNA genes (27). Maintenance of the differentiated state of globin genes may also require *trans*-acting factor(s), as suggested by the loss of human embryonic (but not adult) α-globin-like gene expression on fusion of K562 cells with mouse erythroleukemic cells expressing adult hemoglobin (28). And recently, Emerson and Felsenfeld have partially purified factor(s) that bind to the 5′ flanking region of the chicken adult β-globin gene and confer nuclease sensitivity (29).

In K562 cells, there is no information about the nature of a *trans*-acting factor(s) responsible for the lack of β-globin gene expression. However, recent results in K562 cells, which demonstrate DNase I hypersensitive sites 5′ to the $^{G}\gamma$-, $^{A}\gamma$- and δ-globin genes in K562 chromatin but no such site 5′ to the β-globin gene (30), may be relevant. The lack of coincident δ- and β-globin gene expression could relate in part to factor(s) conferring an active domain to the β-globin gene. In any event, δ- and β-globin genes differ in their requirements for transcriptional activation, and these differences could be reflected in a separation of the onset of transcription of the two genes. The dissociation of δ- and β-globin gene transcription (7) in K562 cells may be indicative of cells arrested in a previously unobserved stage of globin gene activation. Precedent is provided by neoplasms of lymphoid lineage arrested at various stages of differentiation (31). If true, activation of the δ- and β-globin genes would resemble other globin genes in that globin genes are activated developmentally in a 5′ to 3′ direction.

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