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Stable transfer and expression of exogenous human globin genes in human erythroleukemia (K562) cells

(gene transfer)

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ABSTRACT **To study the expression of globin genes in human cells, human ε-globin genes were transferred into a K562 cell line, Bos, which synthesizes very low amounts of ε-globin mRNA. A plasmid (pSV2neo-ε) containing a complete ε-globin gene and 2 kilobases (kb) of 5′ flanking DNA as well as a neomycin-resistance gene and a simian virus 40 origin of replication was transfected into Bos cells; the compound G418, a neomycin analogue, was used to select transformed cells. The presence of unique bands by DNA restriction analysis shows that 11 of 14 of the G418-resistant clones have at least one copy of an integrated ε-globin gene. RNA expression measured by RNA blotting shows significantly more ε-globin mRNA sequences than in untransfected Bos cells in 10 of 11 lines; in most lines, ε-globin mRNA was additionally increased in the presence of hemin. In two lines, ε-globin mRNA expression with hemin was comparable to that of a high ε-globin producing cell line, K562 clone 2. The one G418-resistant line without ε-globin genes had no ε-mRNA expression. The high ε-mRNA expression in several of the lines suggests that exogenous ε-globin genes with only 2-kb 5′ flanking DNA may be sufficient to be appropriately expressed in these homologous erythroid cells. These results have implications for the potential success of transfer of normal human genes to human bone marrow cells as an approach to the treatment of inherited anemias.**

Some of the sequences within and 5′ and 3′ to eukaryotic genes and necessary for their expression have been elucidated during recent years (1–7). In addition, enhancer elements, which can increase transcription of a gene irrespective of their orientation or position with respect to the 5′ and 3′ ends of the gene, have been identified in several systems, including elements flanking the genes as well as within introns of immunoglobulin genes (8–10). The details of how these sequences or other elements interact with cellular factors to control the cell- and tissue-specificity of gene expression *in vivo* is not clear.

The human β-globin gene with only 2 kilobases (kb) 5′ flanking sequence has been shown to be expressed in mouse erythroleukemia cells (MEL cells) after stable transformation (11, 12). More importantly, a marked increase in human β- and ε-globin mRNAs has been demonstrated on addition of compounds that induce endogenous globin gene expression in these cells (11–13). Thus, relatively little DNA is required for expression and induction of globin genes in this erythroid cell line. Tissue-specific expression of transfected genes has also been documented in several other cell types (14–20).

To further explore the role of species- and cell-specific factors in the expression of globin genes, we have transferred human ε-globin genes into a human erythroleukemia cell line (K562 cells) (21–24) and analyzed the expression of these genes. We have used a low ε-globin producing cell line, Bos cells, for this analysis (24). We find that 10 of 11 stable transformants obtained in this system express ε-globin mRNA. In addition, most lines are inducible—i.e., there is an increase in ε-globin mRNA accumulation in the presence of hemin, a compound that increases the rate of hemoglobin accumulation. The results suggest that the DNA present in the recombinant used is sufficient for high level expression of exogenous ε-globin genes in K562 cells. In these experiments, we note significantly higher levels of expression of transfected ε-globin genes than in experiments in which human genes were transferred to MEL cells (11–13). This may be due to species-specific factors that increase expression of the transfected gene. The high level of ε-globin gene expression of several lines in which the ε-globin gene is integrated at different genomic sites in K562 DNA also suggests that specific integration sites are not required for appropriate expression of transfected ε-globin genes in K562 cells, although certain sites give higher levels of expression than others.

MATERIALS AND METHODS

Cell Growth and Gene Transfer. A normal K562 cell line (clone 2) and a low ε-globin producing cell line, K562 Bos, were grown in RPMI 1640 medium with 10% fetal calf serum, as described (24). K562 Bos cells were used in the gene-transfer experiments. For stable transformation, 0.5×10^6 cells per plate were used. Up to 40 μg of DNA was added per plate. Twenty micrograms of plasmid DNA per plate and 10–20 μg of salmon sperm DNA per plate were used. The K562 Bos cells grow in suspension, but the transformations and selections were done in 10-cm plates.

The plasmid used in these experiments (pSV2neo-ε) (Fig. 1) was constructed from plasmids supplied by others: pSV2neo (25), and an ε-globin-containing plasmid, a kind gift from A. Nienhuis. The human ε-globin gene was cloned into pSV2neo in the *Eco*RI site as indicated in Fig. 1. The DNA used for transfection was precipitated in ethanol, rinsed with 70% ethanol, and under sterile conditions under a hood, resuspended in 1 mM Tris·HCl/0.1 mM EDTA, pH 7.6. The DNA was then added to an equal volume of 500 mM $CaCl_2$, and the mixture was added to an equal volume of a balanced salt solution to give a final concentration of 137 mM NaCl/5 mM KCl/0.6 mM $Na_2HPO_4 \cdot 2H_2O$/5.55 mM dextrose/21 mM Hepes buffered to pH 7.12. One milliliter of the final mixture was used on a plate containing 5×10^5 cells. After 24 hr, the

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Abbreviation: kb, kilobase(s).
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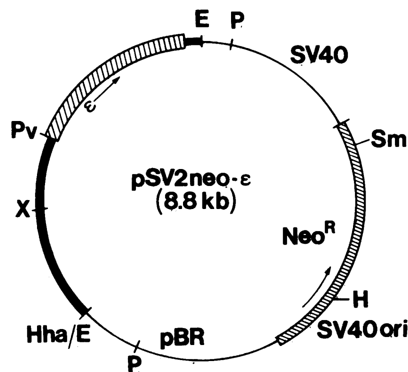

FIG. 1. The pSV2neo-ε vector. The structural ε-globin gene is shown as the hatched rectangular area on the left, while the flanking sequences of the cloned ε-globin gene are indicated by the solid black bars. The neomycin-resistance gene (Neo^R) linked to a simian virus 40 (SV40) origin of replication (ori) is shown as the hatched rectangular area on the right. The *Pst* I (P), *Xba* I (X), *Eco*RI (E), *Hin*dIII (H), *Sma* I (Sm), and one of the *Pvu* II (Pv) and *Hha* I (Hha) sites are indicated. The *Pvu* II/*Eco*RI fragment was the ε-globin probe, and the *Hin*dIII/*Sma* I fragment was the Neo^R gene probe.

precipitate was more easily seen. At this time, cells were replated with fresh medium.

Selection of Transformed Cells. After 24 hr of growth in normal medium, the medium was changed to one containing 600 μg of neomycin analogue (G418) per ml (26). After 4 days, the medium was again changed, and the cells were subjected to 800 μg of G418 per ml. After 5–7 days, the medium was again changed, and 800 μg of G418 per ml was again applied. After an additional 5–7 days, the plates were checked, and if controls showed no viable cells, selection was assumed to be complete and the G418 concentration was decreased to 400 μg/ml. Dead cells were removed using lymphocyte separation medium (Bionetics, Charleston, SC).

Isolation and Analysis of Transformed Cells. Only one-third of the plates inoculated showed G418-resistant cells, and previous experiments with attached cells showed that three colonies on one plate were derived from the same transformation event. Therefore, in these experiments, it was assumed that only one transformation event had occurred on any one plate. Cells from positive plates were grown until $20–50 \times 10^6$ cells accumulated.

DNA and RNA were prepared basically as described (27). The cells were pelleted, rinsed with 20 ml of phosphate-buffered saline (pH 7.2), and repelleted. The cells were then resuspended in 5 ml of 20 mM Tris·HCl, pH 7.6/10 mM EDTA, and 0.25 ml of 20% Sarkosyl was added to lyse the cells. Cesium chloride (5.25 g) was added, and the mixture was gently rotated until all the cesium dissolved. A 3-ml cushion of cesium chloride at 1.35 g/ml in 20 mM Tris·HCl, pH 7.6/10 mM EDTA/0.3% DEPC was placed in an SW 41 tube. The sample was layered on this cushion and spun in a Beckman ultracentrifuge at 30,000 rpm for 20 hr at 15°C. RNA is pelleted and DNA is at the sample–cesium cushion interface. The top of the sample layer was carefully removed and discarded, and the DNA band was collected. The RNA pellet was collected separately. The RNA pellet was suspended in 10 mM Tris·HCl, pH 7.6/1 mM EDTA/0.2% $NaDodSO_4$ in a total vol of 4.5 ml; 0.5 ml of 3 M sodium acetate was added, and the RNA was precipitated by the addition of 2.5 vol of 95% ethanol at −20°C. The DNA was dialyzed against 10 mM Tris·HCl, pH 7.6/1 mM EDTA/0.2% $NaDodSO_4$ with two changes to remove all of the cesium chloride. The DNA was then phenol-extracted and precipitated by the addition of salt and ethanol.

Southern blotting and restriction analyses of DNA were done as described (28, 29). Either a pSV2neo fragment or an ε-globin fragment from pSV2neo-ε was nick-translated (specific activity, $\approx 10^9$ cpm/μg) and used as probe. The neomycin probe used was cleaved from the vector using the enzymes *Hin*dIII/*Sma* I, and the globin probe was removed from the vector with *Pvu* II/*Eco*RI. RNA was analyzed by RNA blotting, as described, using formaldehyde-agarose gels and transfer to nitrocellulose filters (24, 30). S1 nuclease analysis was carried out as described, using end-labeled probes representing the 5′ end of the ε-globin gene (31, 32).

Hemin Induction. Untransformed and transformed cells were analyzed in both the absence and presence of hemin; 20–50 μM hemin was used and gave approximately the same percentages of induction; 50 μM hemin was found to retard cell growth, and, therefore, 20 μM hemin was used in these experiments. The stock hemin solution (Sigma bovine hemin type 1) was prepared by dissolving hemin in 1 M NaOH and adjusted to pH 7.4 with 1 M Tris·HCl. Induced cells were grown in hemin for 4–5 days, and the pellets were red when cells were spun down. The cells were stained for hemoglobin by using benzidine 0.2% (benzidine dihydrochloride dissolved in 0.5 M acetic acid). Less than 10% of the cells were benzidine positive in the absence of hemin, and 60%–80% of the cells were benzidine positive in the presence of hemin.

RESULTS

Transformation of Cells. Fourteen of the 40 plates that received 20 μg of pSV2neo-ε contained growing cells after 3 weeks of exposure to G418. The efficiency of transfection was, therefore, $\approx 1/10^{-7}$ to $1/10^{-8}$ cells per μg of DNA by calcium phosphate precipitation.

DNA Analysis of Transformants. All of the G418-resistant lines demonstrated the presence of neomycin-resistance genes by Southern blotting analysis (data not shown). Eleven of 14 G418-resistant lines studied show evidence for the presence of at least one exogenous ε-globin gene, as indicated by bands absent in the Bos cells. One line, C9, showed no ε-globin bands; two of the 14 lines, A5 and C7, had very faint extra ε-globin bands seen only in some digests, sug-

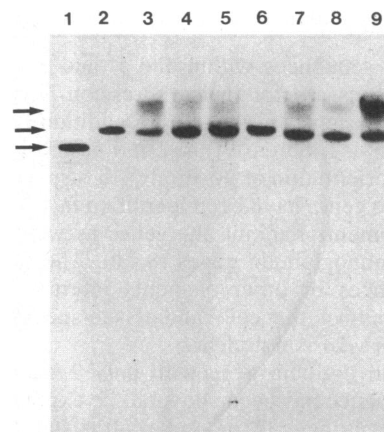

FIG. 2. *Kpn* I digestion of DNA. DNA was isolated from pSV2neo-ε (lane 1), from untransformed Bos cells (lane 2), and from transformed lines (lanes 3–9), cleaved with *Kpn* I, and subjected to agarose gel electrophoresis using a 0.5% gel blotted and hybridized to a ^{32}P-labeled ε-globin gene probe, as described. Plasmid DNA (50 pg) was used in lane 1, while 15 μg of cell DNA was used for the other samples. Lane 2, Bos; lane 3, A6; lane 4, A7; lane 5, A8; lane 6, B1; lane 7, B6; lane 8, B10; lane 9, D4. The lowest arrow points to the plasmid band. The middle arrow points to the endogenous ε-globin gene. The upper arrow indicates the region in which bands larger than the endogenous band are seen that represent integrated ε-globin genes; these are only present in the transformed lines (lanes 3–9).

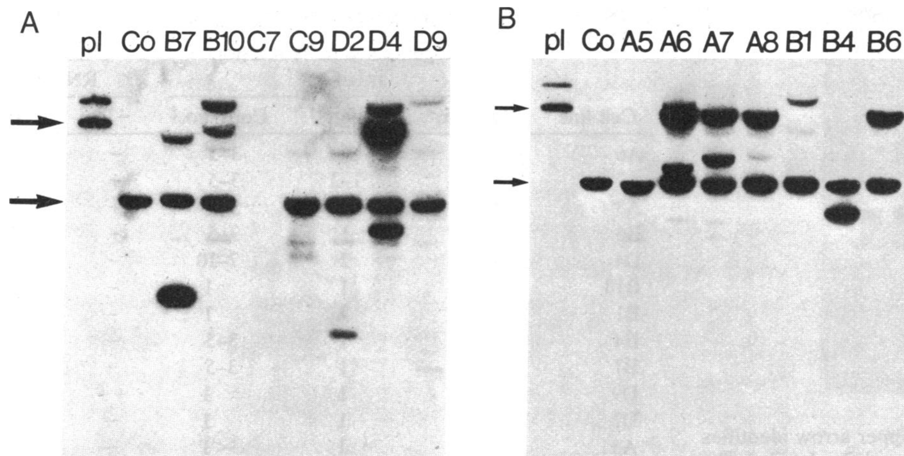

FIG. 3. *Eco*RI digestion of DNAs. DNA from the plasmids (pl), from control untransformed Bos cells (Co), and from the cell lines indicated was digested with *Eco*RI, run on 0.7% agarose gels, and blotted and hybridized to a ^{32}P-labeled ε-globin probe, as described. In these experiments, all of the DNAs were digested with *Eco*RI. The upper arrow is at 8.8 kb and represents the linearized plasmid. The lower arrow, at 3.7 kb, is the endogenous ε-globin DNA band. The additional bands are fragments containing exogenous ε-globin genes integrated into cell DNA. (*A*) B7 to D9. (*B*) A5 to B6.

gesting a mixed population of cells, and they have not been analyzed in detail. The arrangement of exogenous globin genes in the other lines was determined by restriction analysis. The absence of free plasmid in the cell lines was demonstrated using *Kpn* I, an enzyme that does not cleave within the plasmid (Fig. 2). All of the *Kpn* fragments containing ε-globin genes are present in molecules larger than the intact 8.8-kb plasmid, indicating their integration into cell DNA.

To determine the status of the integrated ε-globin copies, digests were prepared with *Eco*RI, *Pst* I, and *Pst* I/*Xba* I (Figs. 3–5). All of the digests show new bands not present in untransfected control Bos cells in most cell lines (Figs. 3–5). The *Eco*RI band containing the endogenous ε-globin gene is 3.7 kb in size (Fig. 3). Bands larger than 3.7 kb indicate the presence of integrated plasmid sequences. Tandem integration or amplification of the pSV2neo-ε plasmid would be expected to lead to an 8.8-kb *Eco*RI fragment, because this fragment is generated by linearizing the intact plasmid. This band is seen in lines A6, A7, A8, B6, B10, and D4 (Fig. 3; Table 1). Additional bands are also seen in several of the lines, both larger and smaller than the endogenous 3.7-kb band, indicating integration of ε-globin gene fragments. Only one of the G418-resistant lines, line C9, has no evidence for ε-globin gene components. This result suggests that only the neomycin-resistance gene was integrated into C9 cell DNA.

Pst I digestion (Fig. 4) also shows the presence of unique bands in many of the clones. The endogenous band containing the ε-globin generated by *Pst* I is >20 kb and is the only band seen in untransformed cells. By contrast, all of the other lines, except C9, contain new bands, A 4.2-kb *Pst* I band containing the ε-globin gene is generated from within the plasmid and is also expected with a tandem array; this band is found in lines A6, A7, A8, B1, B6, B10, and D4 (Fig. 4; Table 1).

Pst I/*Xba* I digestion was done on all the lines that did not contain an intact *Pst* I 4.2-kb band (Fig. 5). All of the lines, except C9, contain bands hybridizing to the ε-globin probe. Line D2, however, does not contain a normal complete *Xba* I/*Pst* I fragment (Fig. 5).

Most of the lines isolated contain between one and five integrated copies of exogenous ε-globin genes except for D4, which shows significantly higher numbers of integrated copies (Figs. 3 and 4; Table 1). D4 shows a large number of tandem copies as well as additional bands; two of these bands may represent the integration sites at the ends of the tandem integration, while other bands indicate additional integration sites.

Expression of ε-Globin Genes in Transformants. RNA from all of the transformed lines shows the presence of neomycin-resistance gene-specific RNA (data not shown). When an ε-globin gene probe is used, there is expression of ε-globin mRNA in 10 of the 11 lines that contain at least one copy of the exogenous ε-globin gene (Fig. 6; Table 1). The only line in which there is no expression is C9. By contrast, the Bos cell line used in these transfections shows little ε-globin mRNA expression in the absence of hemin and an increase, but still only a small amount, in the presence of hemin. In the presence of hemin, there is a significant increase in the amount of ε-globin mRNA expression in 7 of the lines containing exogenous ε-globin genes, while in 3 lines, there is no significant increase. In 2 of the lines, D2 and D9, the amount of ε-globin mRNA accumulated in the presence of hemin is comparable to that in the presence of a high ε-globin producing line, K562 (clone 2) (Fig. 6). In other lines, the

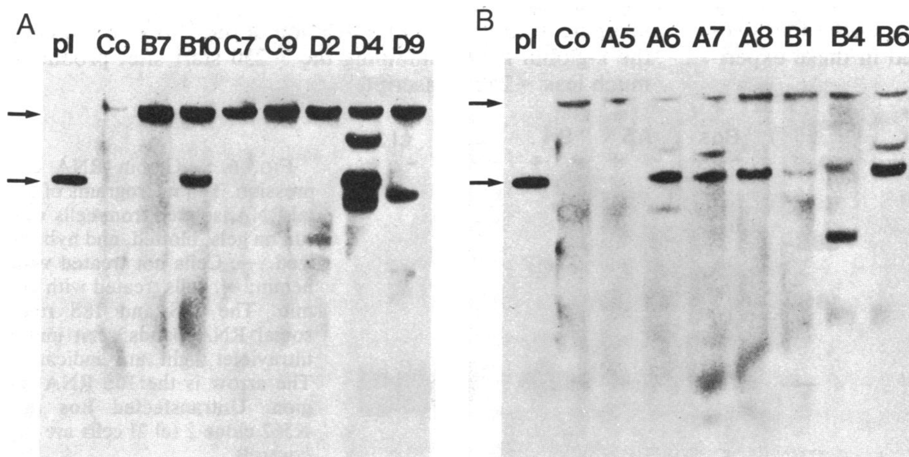

FIG. 4. *Pst* I digestion of DNAs. Upper arrow shows the position of the endogenous ε-globin gene at >20 kb. Lower arrow is at 4.2 kb (the *Pst* I fragment shown in Fig. 1). The additional bands indicate fragments containing exogenous ε-globin genes integrated in the cell DNA. (*A*) B7 to D9. (*B*) A5 to B6.

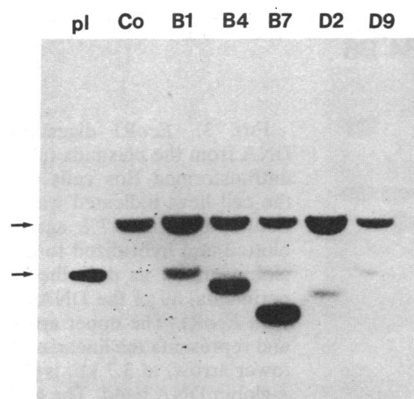

FIG. 5. *Pst* I/*Xba* I digestion of DNAs. Upper arrow identifies the endogenous ε-globin gene. (See Fig. 3 legend for details.) The plasmid (pl) band, indicated by the lower arrow, is the size of the 2.3-kb *Pst* I/*Xba* I fragment containing the entire ε-globin structural gene and its 5′ flanking region (Fig. 1). This fragment is intact in all cell lines shown except D2, which has a 2.0-kb fragment instead of the 2.3-kb fragment.

synthesis and accumulation of ε-globin mRNA is less than in wild type, but in all lines there is significant accumulation of ε-globin mRNA except for C9. There is variability in the relative increase in amount of ε-globin mRNA in both the presence and absence of hemin (Table 1). The amount of neomycin-resistant mRNA does not increase upon hemin induction (data not shown).

S1 Nuclease Analysis. S1 nuclease analysis of RNAs from untransfected Bos cells and three cell lines is shown in Fig. 7, using end-labeled probe derived from an *Mbo* II restriction fragment spanning the 5′ end of the ε-globin gene. A 118-nucleotide fragment is protected if the canonical cap site is used to initiate RNA, while a 310-nucleotide fragment results if an upstream initiation site of −250 is used (33). In Bos cells, in the absence and presence of hemin, there is significantly more initiation at the canonical cap site than the −250 site (Fig. 7). There is also an increase in the presence of hemin similar to that seen by RNA blot analysis (Fig. 6). Similarly, there is increased globin mRNA expression, confirmed by S1 nuclease analysis in lines B4, D2, and A7. There are many more globin mRNA fragments detected by S1 nuclease analysis than in Bos cells in both the presence and absence of hemin (Fig. 7). In all of the lines examined except D2, the ratio of −250 site signal to canonical cap signal is ≈1:20. By contrast, in D2, there is much less −250 signal than canonical cap signal. D2 is the only line in which the exogenous ε-globin gene is deleted in its 5′ flanking region (Fig. 5). These results are consistent with the conclusion that the decreased −250 signal in D2 is due to deletion of the −250 site in this line and support the conclusion that exogenous ε-globin genes are being expressed in these experiments.

Table 1. Gene transfer and expression

Cell line	Exogenous gene restriction fragment			ε-Globin RNA[§]	
	Tandem*	Unique†	Copy no.‡	−H	+H
A6	+	2	3–5	−	+
A7	+	1–2	3–5	+	+
A8	+	1	3–5	+	+
B6	+	1	3–5	−	+
D4	+	2	7–10	−	+
B10	+	1	1	−	−
B1	−	1	1	−	++
B4	−	1	3–5	−	++
B7	−	1	3–5	−	+
D9	−	1	1	++	+++
D2	−	1	1	−	+++
A5	−	1	<1	−	+
C7	−	1	<1	−	+
C9	−	1	<1	−	−
K562 Bos	NA	NA	NA	−	+
K562 cl 2	NA	NA	NA	+	+++

NA, not applicable.
*+ indicates evidence for tandemly inserted copies of intact pSV2neo-ε on the basis of the *Eco*RI and *Pst* I digestions discussed in the text.
†Unique fragments, presumably junctions between exogenous genes and endogenous DNA.
‡Gene copy number.
§RNA expression without hemin (−H) and with hemin (+H).

DISCUSSION

We have demonstrated the stable transfer, integration, and expression of ε-globin genes in an erythroleukemia cell line, K562. One surprising result of these experiments has been the extremely high level of ε-globin mRNA expression in at least two of the transformed lines. It is possible that this is due to the presence of the simian virus 40 enhancer in the construct used. However, the presence of 2.3 kb of plasmid sequences between the enhancer and the ε-globin cap site would be expected to markedly decrease any enhancer effect (34).

Alternatively, the high levels of ε-globin mRNA expression in our experiments could be to reactivation of endogenous ε-globin genes in Bos cells. However, the fact that 10 of 11 colonies transfected with ε-globin genes at different locations all express ε-globin mRNA in various amounts makes this less likely. Spontaneous reactivation of ε-globin genes might be expected to occur in a small percentage of transformants, but not in >90% of the transformants, as seen in our experiments. The one line containing a neomycin-resistance gene and no exogenous ε-globin genes also produced no ε-globin mRNA. In addition, line D2, in which restriction mapping is consistent with deletion of the 5′ region flanking the ε-globin gene containing the −250 start site, produces much less −250 transcript.

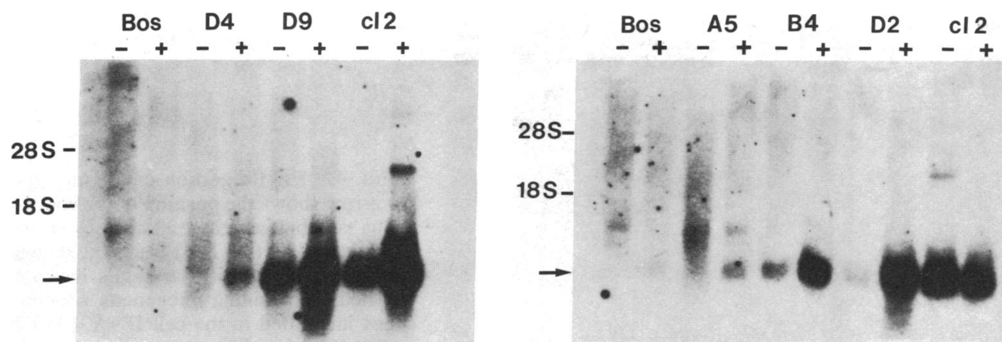

FIG. 6. ε-Globin RNA expression. Ten micrograms of total RNA isolated from cells was run on gels, blotted, and hybridized. −, Cells not treated with hemin; +, cells treated with hemin. The 28S and 18S ribosomal RNA bands seen under ultraviolet light are indicated. The arrow is the 10S RNA region. Untransfected Bos and K562 clone 2 (cl 2) cells are the controls.

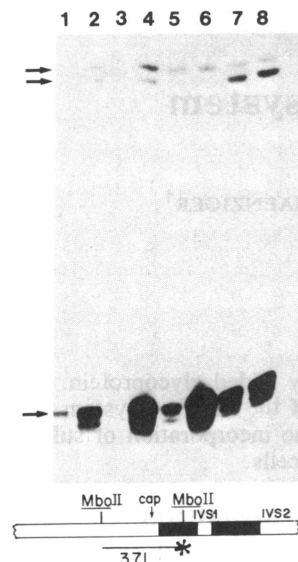

FIG. 7. S1 nuclease analysis of RNAs. RNAs were hybridized to an end-labeled probe spanning the 5' end of the ε-globin gene (33). Upper arrow, pointing to the largest band, identifies the intact probe. Middle arrow shows the 310-nucleotide fragment expected if an upstream site at −250 is used as the cap site; lower arrow shows the 118-nucleotide fragment expected from initiation at the canonical cap. Lanes: 1, Bos (−H); 2, Bos (+H); 3, no DNA; 4, B4 (+H); 5, D2 (−H); 6, D2 (+H); 7, A7 (−H); 8, A7 (+H). IVS1 and IVS2, intervening sequences 1 and 2.

Higher levels of human globin mRNA expression are seen in these experiments using human erythroid cells than when human globin genes are added to MEL cells (11–13). These results could be due to the presence of species-specific factors leading to high ε-globin gene expression in K562 cells. Tissue-specific high level expression has recently been reported of immunoglobulin, insulin, and chymotrypsin 5' regions (15–17, 19). The tissue specificity and high level of expression of ε-globin genes in these studies, and of immunoglobulin, insulin, and chymotrypsin promoters in other recently reported studies (15–17, 19), suggest that maximal expression of transferred genes may depend on the target cells used as well as the chromosomal position of the transferred genes. Our findings as well as those of other studies indicate that these exogenous genes integrated at many different, presumably random, sites can be expressed at high levels, although some sites give higher levels than others. The chromosomal structure at the site of integration of the transferred exogenous genes may determine whether these genes are expressed in appropriate target cells.

The results of gene transfer experiments to date are consistent with the known diverse chromosomal locations of different genes, all of which must be activated in specific tissues. In erythrocytes in normal erythroid differentiation, for example, this activation would include α- and β-globin genes on different chromosomes and genes for enzymes and other proteins on other chromosomes. The factors responsible for the coordinate regulation and expression of these genes remain to be elucidated.

Preliminary analysis shows a relative increase in ε-globin synthesis in the two transfected cell lines with the highest ε-globin mRNA levels (B. P. Alter, personal communication). The high level of expression of human globin genes in human erythroid cells has significant implications for the potential success of experiments whose goal is the cure of inherited anemias in humans by transfer of normal β-globin genes to the bone marrow cells of patients with inherited disorders, such as sickle cell anemia and thalassemia. The high level of expression of transferred genes provides an additional impetus for the development of more efficient vectors for transferring genes into defective cells and for defining new selection systems for providing survival of cells containing the genes of interest. If high level β-globin gene expression can be shown in the bone marrow cells of patients with β-thalassemia, then the use of retroviral vectors with efficient infection of relevant cells, and selectable genes to isolate appropriate transformants both *in vitro* and *in vivo*, may herald success in new attempts at gene transfer in humans.

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