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Regulated expression of genes inserted at the human chromosomal β-globin locus by homologous recombination

(mouse erythroleukemia–human cell hybrids/human chromosome 11/hexamethylenebisacetamide/erythroid differentiation)

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ABSTRACT **We have examined the effect of the site of integration on the expression of cloned genes introduced into cultured erythroid cells. Smithies *et al.* [Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A. & Kucherlapati, R. S. (1985) *Nature (London)* 317, 230–234] reported the targeted integration of DNA into the human β-globin locus on chromosome 11 in a mouse erythroleukemia–human cell hybrid. These hybrid cells can undergo erythroid differentiation leading to greatly increased mouse and human β-globin synthesis. By transfection of these hybrid cells with a plasmid carrying a modified human β-globin gene and a foreign gene composed of the coding sequence of the bacterial neomycin-resistance gene linked to simian virus 40 transcription signals (SV*neo*), cells were obtained in which the two genes are integrated at the β-globin locus on human chromosome 11 or at random sites. When we examined the response of the integrated genes to cell differentiation, we found that the genes inserted at the β-globin locus were induced during differentiation, whereas randomly positioned copies were not induced. Even the foreign SV*neo* gene was inducible when it had been integrated at the β-globin locus. The results show that genes introduced at the β-globin locus acquire some of the regulatory properties of globin genes during erythroid differentiation.**

Mouse erythroleukemia (MEL) cells are transformed lines of erythroid precursors. Treatment of these cells with chemical agents such as hexamethylenebisacetamide (HMBA) causes them to reinitiate a program of erythroid differentiation culminating in terminal cell division and extensive hemoglobin synthesis (1). Several types of studies have demonstrated the utility of the MEL cell system for investigating human globin gene regulation. For example, transfer of human globin genes on intact human chromosomes by cell fusion with MEL cells has shown that the adult human α- and β-globin genes are correctly regulated when the cell hybrids are induced to differentiate (2–5). Activation of human globin genes after transfer to MEL cells can occur even with chromosomes derived from human nonerythroid cells such as diploid fibroblasts. Moreover, the level of expression from the human globin genes in such hybrids is comparable to the level of expression of the endogenous mouse globin genes. Cloned human β-globin genes also are inducible after DNA-mediated gene transfer to MEL cells, but the induction is variable and the level of expression per transferred gene copy is often quite low (6, 7). Insufficient expression of cloned human globin genes also occurs in transgenic mice in which the transferred genes are presumably subject to the full array of developmental controls. A careful study of the expression of human β-globin genes in transgenic mice indicated that the level of tissue-specific expression, normalized for gene copy number, is only a small percentage of that from endogenous mouse globin genes (8). One possible explanation for the differences in expression properties between the human globin genes transferred on intact human chromosomes and the same genes transferred by transfection is that the sites of integration of the transferred DNA copies influence their expression. We have investigated this possibility by comparing the expression of cloned genes inserted by homologous recombination at the human β-globin locus on chromosome 11 and at random sites in a MEL–human cell hybrid. The results show that the integration site can have a strong influence on the regulated expression of genes during erythroid differentiation.

METHODS

Cell Culture and Induction of Differentiation. MEL cells (clone DS19) were grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum as described (9). Hu 11 hybrid cells and transfectants of these cells were propagated in the same medium with additions of the components of the hypoxanthine/aminopterin/thymidine (HAT) selection system (10) or HAT plus antibiotic G418 (400 μg/ml), respectively. Cells were induced to differentiate by growth for 4 days in medium containing 5 mM HMBA.

RNA Analysis. Total cellular RNA was isolated by phenol extraction at 60°C (11). Nuclear and cytoplasmic RNA and poly(A)$^-$ and poly(A)$^+$ RNA were prepared as described (12). S1 nuclease protection experiments were carried out by incubating the indicated DNA probes with RNA at 51°C for 16 hr in 10 μl of 80% (vol/vol) formamide/0.4 M NaCl/1 mM EDTA/0.04 M NaPipes, pH 6.4. The mixture was treated with 300 units of S1 nuclease (Bethesda Research Laboratories) in 90 μl of 0.25 M NaCl/0.03 M NaOAc, pH 4.6/1 mM $ZnSO_4$/5% (vol/vol) glycerol/denatured calf thymus DNA (20 μg/ml) for 45 min at 30°C. S1-resistant DNA fragments were ethanol-precipitated, dissolved, and analyzed by electrophoresis in 6% acrylamide gels containing 8 M urea.

Transcription in Isolated Nuclei. Procedures for isolation of nuclei, *in vitro* transcription, isolation of run-on transcripts, preparation of DNA filters, and hybridization were essentially as described (9, 11) except that each DNA band contained 4 μg of the indicated DNA and hybridization was in 7.5 ml for 48 hr.

RESULTS

Modification of the Human β-Globin Locus. The structure of the modified human β-globin locus that was produced by homologous recombination with a plasmid containing human

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Abbreviations: MEL, mouse erythroleukemia; HMBA, hexamethylenebisacetamide; nt, nucleotide(s).

β-globin sequences (13) is shown in Fig. 1*a*. The cells used in the modification were MEL–human cell hybrids (Hu 11 cells) carrying a portion of human chromosome 11 including the β-globin locus (29). We refer to the intact β-globin gene at the locus as the resident human β-globin gene. Like MEL cells, the Hu 11 hybrid cells can be induced to undergo erythroid differentiation by HMBA, which causes coinduction of mouse α- and β-globin and human β-globin (refs. 3 and 29; also see below). As described by Smithies *et al.* (13), Hu 11 hybrid cells were transfected with a plasmid, pΔβ117, containing 4.6 kb of DNA from the human β-globin locus, including the 5′ part of the human β-globin gene (Δβ) but lacking sequences 3′ of the *Bam*HI site in the second exon. Thus the Δβ gene does not contain either of the recently identified β-globin enhancer sequences, one of which lies within the gene 3′ of the *Bam*HI site and the other of which lies in the 3′ flanking region (8, 15, 16). pΔβ117 also contains the SV*neo* transcription unit [composed of the bacterial neomycin-resistance gene (*neo*) and simian virus 40 transcription signals], conferring resistance to G418, and additional prokaryotic DNA sequences. Homologous recombination between the resident human β-globin locus and the 5′ sequences flanking the Δβ test gene was detected in 0.1–0.3% of G418-resistant transferants. A population of G418-resistant cells was subjected to several rounds of sib selection to isolate a cloned, hybrid cell line in which the structure of the modified locus shown in Fig. 1*a* was proven by Southern (17) blot hybridization of restriction enzyme-digested genomic DNA. The recombination inserted plasmid-derived DNA sequences into the δ–β intergenic region about 4.2 kb 5′ of the resident human β-globin gene. The transcriptional orientation of the inserted Δβ gene is the same as that of the resident β gene. The inserted SV*neo* transcription unit is oriented in the opposite direction.

Expression of the Inserted Δβ Gene. Expression of the truncated Δβ gene was studied before and after HMBA-induced cell differentiation in the cloned line containing the modified locus, in four clones in which the pΔβ117 plasmid was integrated randomly into the genomes of the recipient cells, and in pools of G418-resistant transferants. These pools were derived from several hundred transferants so that the expression of the transferred genes in the pools represents the average of a large number of random integration events. Southern blotting experiments showed that each of the four cloned lines contains a single copy of the plasmid and that the pools contain an average of a single copy per cell (ref. 18 and unpublished results). Analysis of the line containing the modified locus indicated (13) that it contains only the single

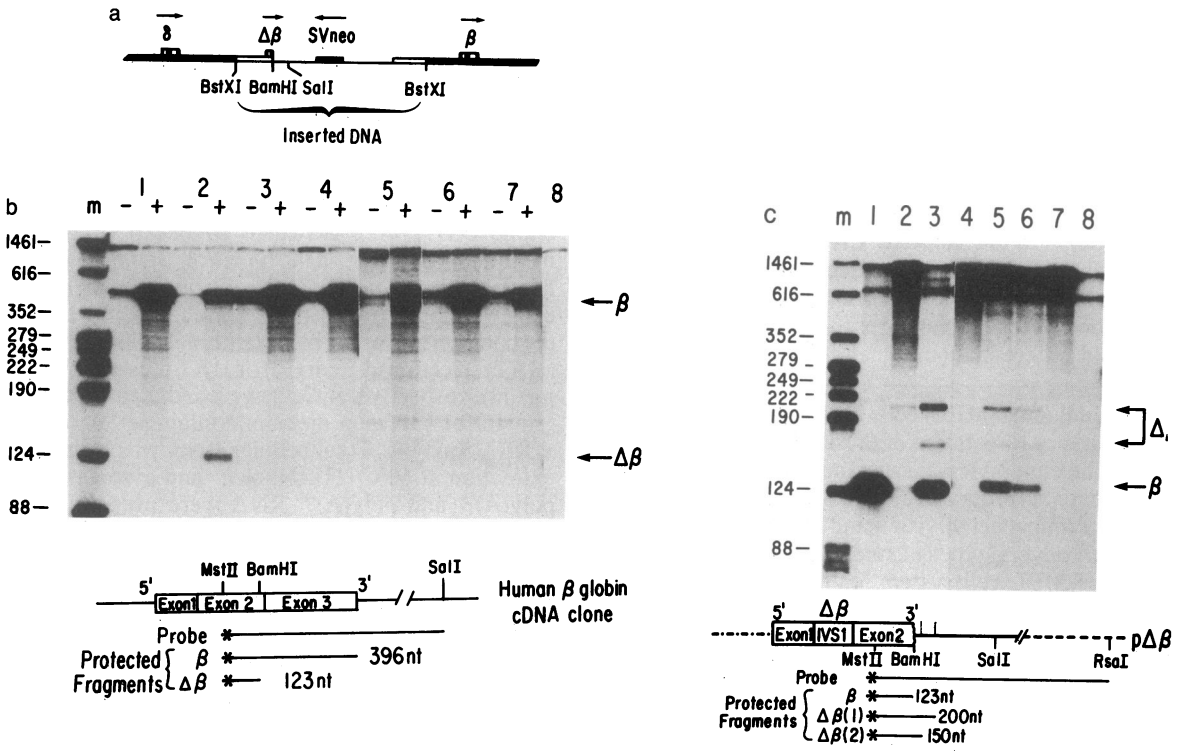

FIG. 1. (*a*) Structure of the modified human β-globin locus. The modification was produced by a homologous recombination between plasmid-derived human β-globin sequences (open bars) on pΔβ117 and resident human β-globin sequences (solid bars) present in a Hu 11 MEL–human hybrid cell. The line indicates sequences mostly derived from pSV2neo (14), from which pΔβ117 was constructed. The position and direction of transcription of the globin (δ, Δβ, and β) and SV*neo* genes are indicated by the raised boxes and arrows. For details see ref. 13. (*b*) S1 nuclease analysis of human β-globin transcripts in Hu 11 transferants. Five micrograms of total RNA from cells grown for 4 days in the presence (+) or absence (−) of 5 mM HMBA was analyzed with the human β-globin cDNA probe, 3′-end-labeled at the *Mst* II site as illustrated. The human β-globin cDNA probe was prepared from pB6-6, a gift from B. Forget (Yale University). pB6-6 DNA was digested with *Mst* II, end-labeled with reverse transcriptase, and digested with *Sal* I, and the 1.2-kilobase (kb) *Mst* II–*Sal* I fragment was isolated from an agarose gel after electrophoresis. Lanes: 1, Hu 11 parent cells; 2, cloned Hu 11 transferant containing the modified globin locus (colony P, ref. 13); 3–6, cloned Hu 11 transferants with pΔβ117 integrated randomly; 7, large pool of Hu 11 transferants; 8, MEL cells treated with HMBA; m, marker DNA fragments prepared by end-labeling *Sau*96I-digested pBR322. Lengths of marker fragments are given in nucleotides (nt) at left. (*c*) Structure and cellular location of Δβ transcripts. RNA was analyzed by S1 nuclease protection with an *Mst* II–*Rsa* I fragment from pΔβ117, 3′-end-labeled at the *Mst* II site as illustrated. This probe was prepared by digesting pΔβ117 with *Mst* II, end-labeling with reverse transcriptase, purifying the 4.1-kb labeled DNA fragment, and digesting it with *Rsa* I. Except where indicated total RNA was isolated from the Hu 11 transferant with the modified globin locus after treatment for 4 days with 5 mM HMBA. Lanes: 1, HMBA-treated Hu 11 parent cells; 2, untreated cells; 3, HMBA-treated cells; 4, poly(A)$^-$ RNA; 5, poly(A)$^+$ RNA; 6, cytoplasmic RNA; 7, nuclear RNA; 8, *Escherichia coli* tRNA. IVS1, intervening sequence (intron) 1.

copy of the plasmid integrated at the β-globin locus. Transcripts of the β genes were assayed by S1 nuclease protection with a human β-globin cDNA fragment that detects the transcripts produced from the inserted Δβ gene and the full-length human β-globin mRNA produced from the resident gene (Fig. 1*b*). Expression of the resident human β-globin gene was strongly induced during HMBA treatment of the parental hybrid cells (Fig. 1*b*, lanes 1) and of all transferants (lanes 2–7). Δβ transcripts were detected at a low level in the transferant pool before differentiation, but they were decreased to extremely low levels after HMBA treatment (lanes 7). These transcripts were not detected either before or after HMBA treatment of the four cloned transferants with randomly integrated copies. However, Δβ transcripts were strongly induced during HMBA treatment of the line with the modified locus (lanes 2).

Because the Δβ gene lacks the normal globin polyadenylylation signal, we wished to determine the structure and cellular location of the Δβ transcripts. S1 nuclease protection experiments were performed with a 3′-labeled DNA probe derived from the pΔβ117 plasmid (Fig. 1*c*). The line containing the modified β-globin locus produced three β-globin-related transcripts that protected the probe. The protected fragment of 123 nt terminating at the *Bam*HI site in exon 2 is produced by hybridization of the labeled probe to full-length β-globin transcripts from the resident gene. The 200-nt and 150-nt protected fragments are derived from Δβ transcripts that extend 75 and 25 nt *beyond* the *Bam*HI site in exon 2 to sites in pBR322 DNA. These Δβ transcripts were inducible (Fig. 1*c*, lanes 2 and 3) and accumulated in the cytoplasm (compare lanes 6 and 7). Both of these transcripts were polyadenylylated as judged by retention on oligo(dT)-cellulose (lane 5). The sites in pBR322 detected in the S1 nuclease protection experiments could represent either 3′ termini of the Δβ transcripts or sites of splicing. Blot hybridization of electrophoretically fractionated RNA showed that the line containing the modified locus produces a β-globin-related transcript of about 1200 nt long in addition to normal human β-globin RNA (data not shown), suggesting that the Δβ transcripts are terminated more than 800 nt downstream from the Δβ gene. Thus, although the exact structure of the two Δβ transcripts remains to be determined, it seems probable that both transcripts are spliced at cryptic sites in the plasmid sequences and that they are terminated at a polyadenylylation signal in the late region of simian virus 40 DNA.

The S1 nuclease protection experiments indicated that, in the cell line with the modified locus, transcription of the Δβ gene proceeds at least some distance into and most likely through the *Bam*HI–*Sal* I fragment of pBR322. We therefore incorporated this fragment into M13 vectors and used it to assay transcription of the Δβ gene in nuclear run-on transcription experiments. In the same experiments a fragment of β-globin intron 2 was used to assay transcription of the resident human β-globin gene. Transcription of the Δβ and β genes in the line with the modified locus was increased markedly during HMBA-induced differentiation (Fig. 2*a*). Thus the accumulation of Δβ transcripts occurring during cell differentiation of this line is due at least in part to increased transcription. Taking account of the sizes of the DNA fragments used to assay transcription of each gene, we estimated that transcription in the Δβ gene integrated at the locus is about 25% that of the neighboring, intact human β gene. Transcription of the Δβ gene was not detectable either before or after HMBA-induced differentiation of transferants containing randomly integrated copies (Fig. 2*b*).

Inducible Expression of a Foreign Gene Inserted at the β-Globin Locus. Expression of the SV*neo* gene is expected in all transferants because the cells are resistant to G418. We were, however, interested to see how the SV*neo* gene inserted into the β-globin locus would respond during HMBA-induced differentiation. S1 nuclease protection experiments were performed with a 5′-labeled DNA probe from the SV*neo* gene to assay for SV*neo* transcripts before and after cell differentiation (Fig. 3). Remarkably, SV*neo* transcripts were strongly induced during differentiation of the line with the modified locus (Fig. 3, lanes 2). In contrast, these transcripts were actually decreased in amount after HMBA treatment in all four clones with randomly integrated copies (lanes 3, 4, 6, and 7)

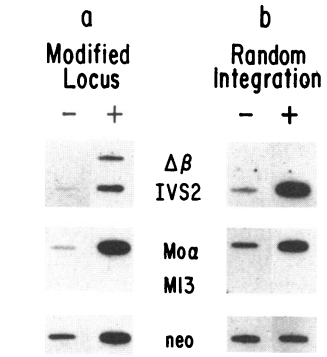

FIG. 2. Nuclear run-on transcription of human β-globin and SV*neo* genes in Hu 11 transferants. [α-^{32}P]UTP-labeled nuclear run-on transcripts were prepared from nuclei isolated from cells grown for 4 days in the presence (+) or absence (−) of 5 mM HMBA. Approximately 4 × 10^7 cpm of each labeled RNA preparation was hybridized to a single nitrocellulose filter on which bands of the indicated DNA probes were adsorbed. Additional DNA bands, not shown here, were also present on the filter. Autoradiographic exposure time was 4 days. (*a*) Labeled nuclear run-on RNA from the Hu 11 transferant containing the modified globin locus was hybridized. Δβ transcription was assayed with the anticoding strand (relative to Δβ; see Fig. 1) of the 276-base-pair *Bam*HI–*Sal* I fragment of pBR322 cloned in M13 mp19. Transcription of the resident human β-globin gene was assayed with the anticoding strand of the 345-base-pair *Rsa* I–Hae III fragment of human β-globin intron 2 (IVS2) cloned in M13 mp8, a gift from J. Metherall (Yale University). SV*neo* transcription was assayed with the anticoding strand of the 1.3-kb *Hin*dIII–*Sma* I fragment of pSV2neo (14) cloned in M13 mp18. Mouse α-globin (Moα) transcription was measured with pZMαG10 (19), a mouse α-globin cDNA recombinant. M13 mp18 DNA was used as a negative control. (*b*) Labeled RNA from the Hu 11 transferants containing randomly integrated copies was hybridized as in *a*.

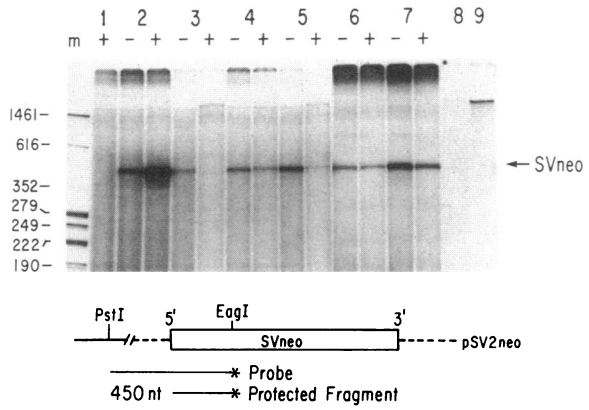

FIG. 3. S1 nuclease analysis of SV*neo* transcripts in Hu 11 transferants. Forty micrograms of total RNA from cells grown for 4 days in the presence (+) or absence (−) of 5 mM HMBA was analyzed with the SV*neo* probe, 5′-end-labeled at the *Eag* I site as illustrated. The probe was prepared by digesting pSV2neo with *Eag* I, labeling with polynucleotide kinase and [γ-^{32}P]ATP, and digesting with *Pst* I. Lanes: 1, Hu 11 parent cells; 2, Hu 11 transferant with the modified locus; 3, 4, 6, and 7, cloned Hu 11 transferants with pΔβ117 integrated randomly; 5, large pool of Hu 11 transferants; 8, *E. coli* tRNA; 9, probe.

and in the large pool of transferants (lanes 5). Down-regulation of SV*neo* transcripts also occurred during HMBA treatment of two additional pools of transferants (data not shown).

Transcription of the SV*neo* gene was assayed directly by nuclear run-on experiments. Transcription of the SV*neo* gene in the line with the modified locus was markedly increased after HMBA treatment (Fig. 2*a*), indicating that accumulation of these transcripts in this line is due at least in part to an increase in transcription of the SV*neo* gene integrated at the β-globin locus. In transferants containing randomly integrated copies, transcription of the SV*neo* gene was unaffected by HMBA treatment (Fig. 2*b*). Thus the observed reduction in the level of SV*neo* transcripts after differentiation of the lines containing randomly integrated copies appears to be due to posttranscriptional control. In other experiments, we found that several endogenous genes, including c-*myc* and those encoding nuclear protein p53, β-actin, and histones H1 and H3, that are constitutively expressed in MEL cells are down-regulated during terminal differentiation. In some of these cases *in vitro* nuclear run-on experiments indicated that control occurs at the posttranscriptional level (ref. 20; A.K.N. and A.I.S., unpublished results). Whether these posttranscriptional effects are due to changes in nuclear processing or transport or due to changes in mRNA stability remains to be determined. Bastos and Aviv (21) have suggested on theoretical grounds that changes in stability of non-globin mRNAs are necessary to achieve the high level of accumulation of globin mRNA seen in terminal erythroid cells.

Expression of the Resident β-Globin Gene in the Modified Locus. We were particularly interested in determining whether the expression of the resident human β-globin gene was affected by insertion of foreign DNA nearby in the β-globin locus. Because the amount of globin RNA produced after cell differentiation can vary in different experiments and in individual clones due to differences in the proportion of differentiated cells, we assayed mouse and human β-globin RNAs simultaneously with two probes of the same specific radioactivity. Fig. 4 shows an S1 nuclease protection analysis of the RNAs. After differentiation, the parental Hu 11 hybrid cells contained equal amounts of mouse and human β-globin RNAs (lanes 1). G418-resistant transferants containing randomly integrated copies of the pΔβ117 plasmid exhibited normalized levels of human β-globin RNA that were 90–200% of the level in the parent cells (lanes 3–7). However, in the cell line in which pΔβ117 was inserted at the human β locus, the production of human β-globin RNA was reduced to 20% of the parental level (lanes 2). Although a larger number of homologous insertion events must be studied before we can draw a firm conclusion regarding this quantitative effect, the results suggest that insertion of foreign DNA at the globin locus reduces expression of the neighboring β-globin gene. The effect could be due to disruption of the 5′ DNA sequences flanking the resident β-globin gene or due to the presence of foreign DNA including prokaryotic sequences. Others have noted an effect of plasmid sequences on the expression of exogenous β-globin genes introduced at random sites in the germ line of mice (22).

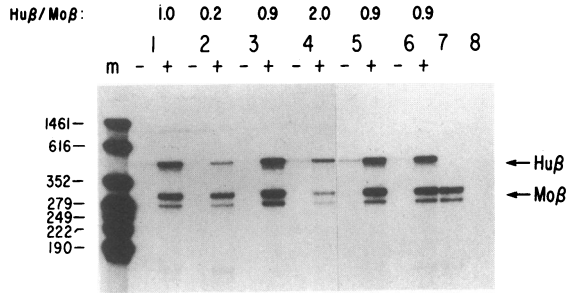

FIG. 4. Ratio of human and mouse β-globin mRNAs in Hu 11 parent cells and transferants. Five micrograms of total RNA from cells grown for 4 days in the presence (+) or absence (−) of 5 mM HMBA was analyzed simultaneously with human and mouse β-globin cDNA probes. The human β-globin cDNA probe was as described in the legend to Fig. 1. The mouse β-globin cDNA probe was prepared by digesting pMβG9 (19) with *Bam*HI, labeling with reverse transcriptase, and digesting with *Hha* I. The labeled probes were adjusted to the same specific radioactivity by addition of unlabeled, restriction enzyme-digested plasmids. The intensity of the autoradiographic bands for induced (+) samples was measured with a Cambridge Instruments Quantimet 970 analyzer and the human/mouse β-globin RNA (Huβ/Moβ) ratios were calculated and are given at the top of the figure. Lanes: 1, Hu 11 parent cells; 2, Hu 11 transferant with the modified locus; 3–6, cloned Hu 11 transferants with pΔβ117 integrated randomly; 7, HMBA-treated MEL cells; 8, *E. coli* tRNA.

DISCUSSION

The results reported here indicate that genes introduced at the β-globin locus exhibit transcriptional regulation in differentiating erythroid cells that is not exhibited by the same genes located at random positions. Regulatory sequences have been reported to be present both 5′ and 3′ of the translation initiation site in the human β-globin gene (23, 24). Consequently, a part of the observed induction of Δβ transcripts may be due to induction of globin-specific transcription factors able to interact directly with the regulatory sequences present in the 5′ region of the Δβ gene. However, the direct effects of such factors cannot explain many of our results. Thus the Δβ transcripts were not induced when the gene was integrated randomly. Very likely the lack of inducible expression of Δβ at random sites is largely due to the absence of enhancer sequences known to be present 3′ of the *Bam*HI site in the human β-globin gene (15, 16, 25), since inducible expression of a complete human β-globin gene in MEL cells has been observed in many cases. We also found that even the foreign gene SV*neo* is inducible when it is integrated at the β-globin locus. These results imply a mechanism that can confer regulated expression on all three of the genes studied. One possible explanation for the observed inductions is that expression of all three genes is influenced by the enhancer sequences lying within and 3′ of the resident human β-globin gene. Another possibility is that the inductions are related to the fact that the β-globin locus in Hu 11 cells is part of an active chromatin domain. Many studies have indicated that the chromatin structure in the vicinity of active genes differs from that near inactive genes (26). Local DNase-hypersensitive sites exist near individual active human globin genes. There also are distal hypersensitive sites, lying 5′ of the ε-globin gene and 3′ of the β-globin gene, that appear to be present whenever one or more of the individual genes in the β-globin complex is expressed, and it has been suggested that these sites define an active globin chromatin domain (27). Grosveld *et al.* (28) reported that regions of DNA including these sites are important for achieving high-level, erythroid-specific expression of human β-globin in transgenic mice. Further experiments are needed to assess the relative contributions of the enhancer sequences and other putative cis-acting elements to the regulated expression of genes targeted to the β-globin locus.

Our findings may also help to explain why the expression properties of human β-globin genes introduced as cloned DNAs in MEL cells and mouse embryos differ from those of the same genes on intact human chromosome 11 in MEL–human cell hybrids. Regulated, tissue-specific expression of cloned human β-globin genes has been observed after transfer to MEL cells and mouse embryos, but the degree of regulation is variable and in some clones it does not occur (6–8). Furthermore, in most instances the amount of expres-

sion per transferred gene copy is only a small fraction of that of the endogenous mouse globin genes. In contrast, the human β-globin gene expressed from an intact chromosome 11 in MEL–human cell hybrids is highly inducible and its expression from a single copy is comparable to that of the endogenous mouse β-globin gene (ref. 29 and Fig. 4). Our results suggest that a high level of expression can be conferred on a transferred β-globin gene by targeting it to the β-globin locus. Nuclear run-on transcription measurements indicated that the Δβ gene positioned at the locus is transcribed with about 25% the efficiency of the resident β-globin gene. This level is much higher than that obtained from a single copy of an intact β-globin gene integrated at random sites in MEL cells. The level might be still higher if enhancer sequences 3′ of the *Bam*HI site in the second exon were included or if potential inhibitory sequences in the plasmid DNA were removed.

Whatever may be the precise mechanism causing regulation of genes inserted at the β-globin locus, our results have implications for future attempts at somatic gene therapy of β-hemoglobinopathies. Successful gene therapy of these disorders will require that newly introduced β-globin genes be expressed at a high level, in amounts stoichiometric with α chains and properly controlled. The results reported here suggest that one way to achieve these goals is to target the inserted genes to the β-globin locus in hematopoietic cells. Alternatively, if all of the relevant regulatory sequences at the β-globin locus can be identified, it may be possible to incorporate them into appropriate expression vectors and thereby achieve precisely controlled expression of genes inserted at random sites.

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