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Expression of Human β-Globin Genes in Transgenic Mice: Effects of a Flanking Metallothionein-Human Growth Hormone Fusion Gene

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In an attempt to place a human β-globin gene in an open chromatin domain regardless of its site of integration in the mouse genome, we microinjected into fertilized mouse eggs a construct in which the human β-globin gene and a mouse metallothionein-human growth hormone fusion gene were juxtaposed and oriented in opposite directions. Mice that developed from injected eggs and that grew larger than normal were analyzed for human β-globin mRNA. The globin genes were not expressed in erythroid tissue but were expressed with the same tissue specificity as metallothionein-human growth hormone. These results suggest that sequences which control metallothionein-human growth hormone gene expression are capable of stimulating the expression of a flanking gene in an orientation-independent and tissue-specific manner. As a control for this experiment, we deleted the metallothionein-human growth hormone transcription unit and noted that the human β-globin gene then was expressed at high levels with erythroid tissue specificity.

The β-globin genes of several species have been stably introduced into the germ line of mice. However, normal levels of erythroid-specific expression have been difficult to achieve. Wagner et al. (19) first introduced human β-globin (hβG) genes into fertilized mouse eggs and found that although the genes were stably integrated into the germ line, no globin mRNA or protein could be detected in adult mice which developed. Wagner et al. (20) microinjected rabbit β-globin genes into fertilized mouse eggs, and although they reported that rabbit globin could be detected in blood of adult mice, rabbit β-globin mRNA was not quantitated and tissue specificity was not examined. Costantini and Lacy (8) and Lacy and co-workers (12) also introduced rabbit globin genes into fertilized mouse eggs and analyzed expression in the animals that developed. The genes were stably integrated into the germ line but were expressed only at a low level in inappropriate tissues. More recently, this same group observed erythroid-specific expression of a hybrid globin gene composed of mouse β^{maj} 5′ and human β3′ sequences (4). However, the level of globin mRNA was low (2.0% of normal at best), and globin protein could not be detected.

One possible explanation for the lack of or low-level expression of globin genes in transgenic mice is that the genes integrate into sites which are not organized into open chromatin domains (22) in adult erythroid tissues. If the injected sequences themselves are unable to organize an open domain, then the globin gene may be inaccessible to erythroid-specific regulators. In an attempt to place an hβG gene (14) in an open chromatin domain (17) regardless of its site of integration in the mouse genome, we microinjected into fertilized mouse eggs a construct in which the hβG gene was juxtaposed to a mouse metallothionein-human growth hormone (MThGh) fusion gene which is expressed well in transgenic mice (15). Large mice that developed from injected eggs then were selected and analyzed for hβG mRNA.

MATERIALS AND METHODS

Plasmid construction and microinjection. An *Hpa*I-*Xba*I fragment containing the *h*βG gene (13) was digested with *BAL* 31 to remove approximately 500 base pairs (bp) of DNA from each end. After filling in the ends with Klenow fragment of DNA polymerase, the deleted fragments were ligated into the filled-in *Bst*EII site of MThGH plasmid 112 (15) and transformed into *Escherichia coli*. A clone which contained approximately 360 bp of globin 5′-flanking and 1,100 bp of globin 3′-flanking DNA was chosen for injection. DNA was digested with *Nru*I and *Kpn*I or with *Sst*I and *Kpn*I, and the 7.3- or 3.5-kilobase fragments, respectively, were separated from other fragments by gel electrophoresis. The DNA was purified, and approximately 600 copies were microinjected into male pronuclei of F2 hybrid eggs obtained by mating C57 × SJL parents as described by Brinster et al. (R. L. Brinster, H. Y. Chen, M. Traumbauer, M. K. Yagle, and R. D. Palmiter, Proc. Natl. Acad. Sci. U.S.A., in press). Pups which contained the injected sequences were identified by dot hybridization of DNA obtained from tail biopsies (Brinster et al., in press). The nick-translated probe used for hybridization was either pBR322 or a 287-bp *Dde*I fragment which contained the 3′ untranslated sequence of the hβG gene and does not cross-hybridize with mouse globin sequences.

Animal treatment and sample preparation. Mice which contained the injected sequences were maintained on zinc water (25 mM $ZnSO_4$) for 2 weeks before sacrifice and made anemic by injection of phenylhydrazine (6). For S1 analysis, RNA was extracted from frozen tissue (except for blood) essentially as described by Chirgwin et al. (7) and further purified by proteinase K digestion, phenol-chloroform ex-

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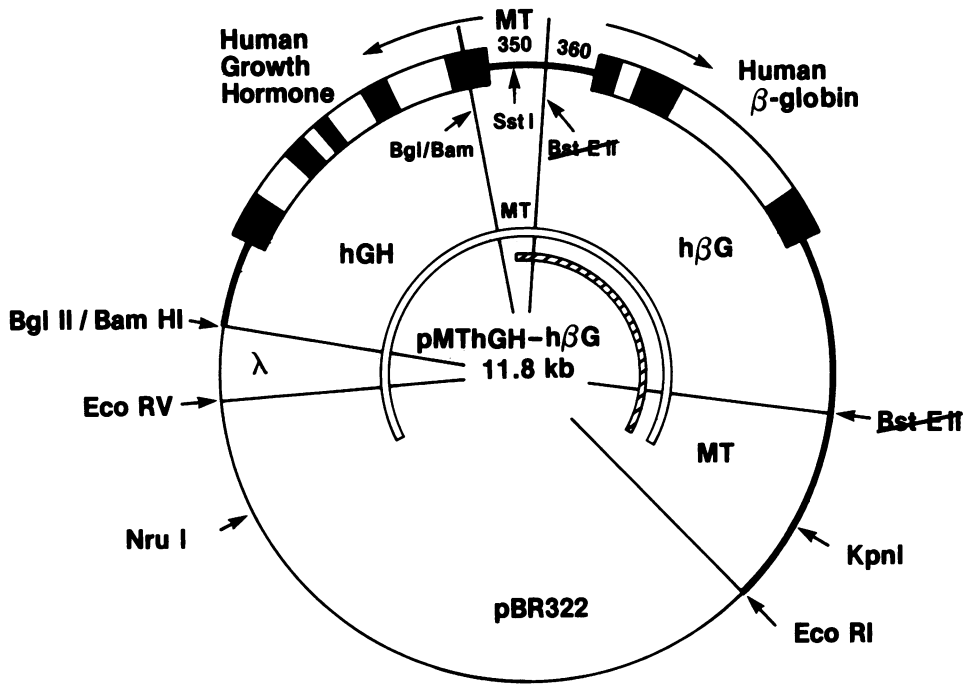


FIG. 1. Structure of the MThGH-hβG plasmid. An hβG gene containing approximately 360 bp of 5'-flanking sequence and 1,100 bp of 3'-flanking sequence was inserted into the filled-in *Bst*EII site of MThGH plasmid 112 (15). The large solid and open boxes represent exons and introns, respectively, in the MThGH and hβG genes. Thick lines represent sequences which flank the 5' and 3' ends of the two genes, and the thin line represents vector sequences. The open semicircular box marks the *Nru*I-*Kpn*I fragment containing both genes that was injected into fertilized eggs. The hatched semicircular box marks the injected *Sst*I-*Kpn*I fragment that contains only the hβG gene plus 200 bp of MT-I flanking sequence at the 5' end and 300 bp of MT-I flanking sequence at the 3' end.

traction, and ethanol precipitation. Blood was collected from the tail vein, and cells were washed with phosphate-buffered saline and lysed in 4 ml of sodium dodecyl sulfate (SDS)-proteinase K as described by Brinster et al. (in press). RNA was then isolated by phenol-chloroform extraction and ethanol precipitation. For solution hybridization with oligonucleotides, 100-mg portions of each tissue were homogenized in SDS-proteinase K, and total nucleic acids were isolated by phenol-chloroform extraction and ethanol precipitation.

S1 analysis. Total cellular RNA (25 μg) from each tissue was hybridized to 5'- or 3'-end-labeled, strand-separated probes for 30 min in 25 μl of a solution containing 1.0 M NaCl, 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, and approximately 0.05 pmol (~100,000 dpm) of probe. After hybridization, the samples were digested with 400 U of S1 nuclease (Sigma Chemical Co.) per ml for 2 h at 20°C in 0.3 ml of 0.28 M NaCl–0.05 mM sodium acetate (pH 4.6)–4.5 mM $ZnSO_4$–20 μg of denatured salmon sperm DNA per ml, then extracted with phenol-chloroform, ethanol precipitated, suspended, and analyzed on an 8% urea–polyacrylamide denaturing gel.

Solution hybridizations. Ten picomoles of oligonucleotides complementary to bases +78 to +98 of hβG mRNA or bases +80 to +100 of mouse β^t mRNA was 5' end labeled in a 10-μl reaction containing 50 mM Tris hydrochloride (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 20 pmol of [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear Corp.), and 1 U of T4 polynucleotide kinase (P-L Biochemicals, Inc.). The labeled products were isolated by electrophoresis on a 12% polyacrylamide gel and eluted in 1.0 ml of 0.2× SET (1× SET is 1% SDS, 10 mM Tris hydrochloride, and 5 mM EDTA; pH 7.5). hβG and mouse β-globin (mβG) RNAs were quantitated by a modification of the procedure of Durnam and Palmiter (9). Briefly, 5,000 cpm of the labeled oligonucleotides were hybridized to total nucleic acid samples for 16 h at 45°C in 30 μl of a solution containing 1.0 M NaCl, 33 mM Tris hydrochloride, 6.6 mM EDTA (pH 7.5), and 0.2% SDS. (The two oligonucleotides differ by 5 bases and hybridize specifically to the appropriate mRNA under these conditions.) After overnight hybridization, the samples were digested with 8.0 U of S1 nuclease (Bethesda Research Laboratories, Inc.) in 1.0 ml of a solution containing 0.3 M NaCl, 30 mM sodium acetate (pH 4.6), 3.0 mM zinc acetate, and 100 μg of undenatured herring sperm DNA per ml, precipitated with trichloroacetic acid, filtered onto glass fiber filters, and counted in a liquid scintillation counter. These values then were compared to a standard curve generated by hybridizing the labeled oligonucleotides to various amounts of M13 clones carrying sequences complementary to the oligonucleotides.

Primer extensions. Approximately 0.05 pmol (~200,000 dpm) of the 5'-end-labeled oligonucleotide(s) was hybridized to total reticulocyte RNA for 30 min at 45°C in 50 μl of a mixture containing 1.0 M NaCl, 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 0.2% SDS, and 20 μg of tRNA. After hybridization, the samples were ethanol precipitated, washed with 70% ethanol, dried, suspended, and incubated at 37°C for 30 min in 20 μl of a solution containing 50 mM Tris hydrochloride (pH 8.2), 6 mM $MgCl_2$, 10 mM dithiothreitol, 100 mM NaCl, 1 mM each of dATP, dCTP, dGTP, and TTP, 20 U of RNasin (Promega Biotec), and 15 U of reverse transcriptase (Boehringer-Mannheim Biochemicals). Reactions were stopped by phenol-chloroform extraction, ethanol precipitated, dried, suspended, and analyzed by electrophoresis on urea-polyacrylamide denaturing gels.

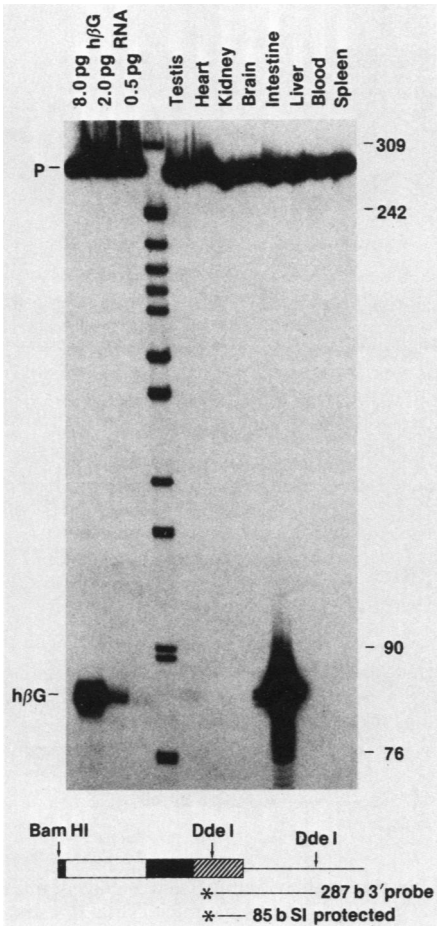


FIG. 2. S1 nuclease assay of total cellular RNA isolated from various tissues of a representative MThGH-hβG transgenic mouse. Total cellular RNA (25 μg) from seven tissues of MTHGH-hβG mouse 21-9 was hybridized to the 3'-end-labeled, strand-separated probe illustrated beneath the figure. S1-protected DNA then was analyzed on an 8.0% urea–polyacrylamide gel. The hβG RNA used as a control was isolated from reticulocytes of a patient with sickle cell anemia and quantitated by solution hybridization to an hβG oligomer as described in the text. Markers are 3'-end-labeled *Hpa*II fragments of pBR322. A schematic representation of the 3' end of the hβG gene is illustrated below the figure. Solid and open boxes mark exons and introns, respectively, and the hatched box represents 3' untranslated sequence. The single-stranded probe and S1-protected product are illustrated below the gene.

RESULTS

An hβG gene with approximately 360 bp of 5'-flanking and 1,100 bp of 3'-flanking sequence was inserted into the *Bst*EII containing the hβG and MThGH genes (open semicircle in Fig. 1) then was isolated and microinjected into fertilized eggs. Of 11 transgenic mice which contained the injected DNA, 7 grew significantly larger than their litter mates which did not contain the genes. The diet of these animals was supplemented with 25 mM $ZnSO_4$ in water to stimulate the MT promoter (15), and they were made anemic by phenylhydrazine injection (6) to elevate circulating reticulocyte levels. RNA then was extracted from blood and six other tissues and analyzed for hβG RNA by an S1 nuclease protection assay (2). Figure 2 illustrates a representative result obtained from the large mice. Surprisingly, the highest levels of hβG RNA were found not in reticulocytes or anemic spleen (an erythroid organ in adult mice) but in intestine. Six of the seven large mice expressed the hβG gene with a similar tissue distribution; the seventh mouse did not express the gene in any tissue. An analysis of intestinal RNA from all 11 transgenic mice is illustrated in Fig. 3A and B. Both the 5' and 3' ends of hβG RNA were the same as those detected in human reticulocytes.

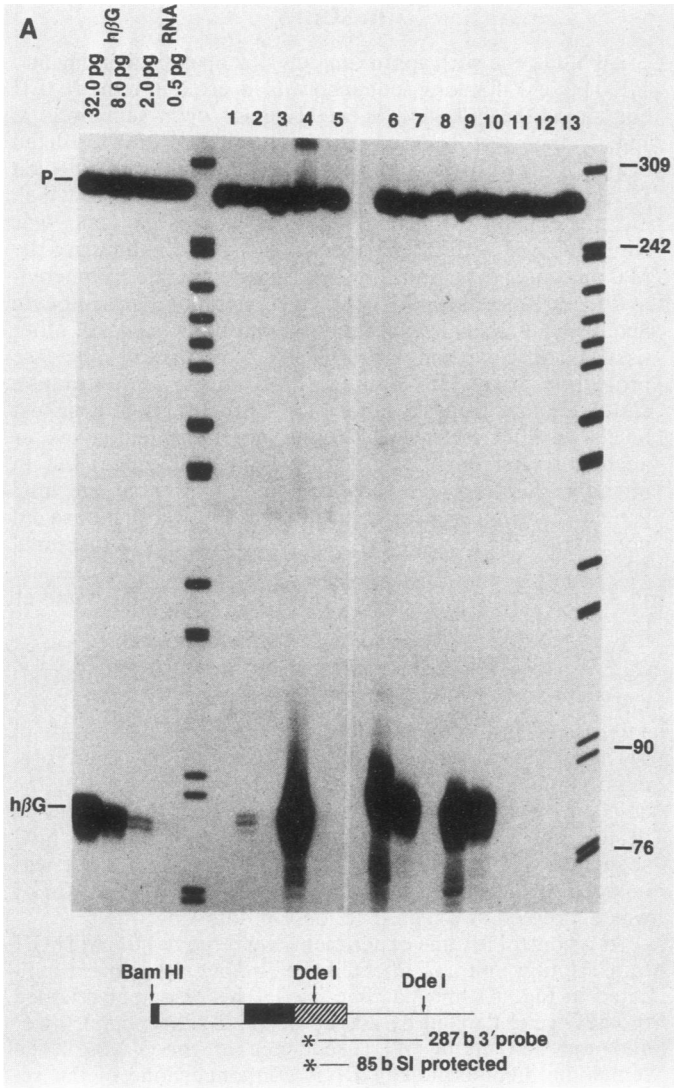
We also assayed most of the tissues of four of the seven large mice for both endogenous metallothionein (MT-I) mRNA and MThGH mRNA. The highest levels of hβG RNA were found in tissues which also synthesized high levels of MThGH and endogenous MT-I RNA (Table 1). Therefore, expression of the hβG gene appears to be stimulated by the adjacent metallothionein promoter. In addition, expression of the MThGH gene may have been inhibited by hβG sequences in the liver because, although MT-I RNA was induced to high levels in both intestine and liver, MThGH was expressed at a high level only in intestine.

As a control for this experiment, we removed the MThGH transcription unit and injected the *Sst*I-*Kpn*I fragment indicated in Fig. 1 (thin, hatched box). This fragment retained the hβG gene flanked by 200 bp of MT-I sequence at the 5' end and 300 bp of MT-I sequence at the 3' end. The remaining metallothionein DNA contained none of the sequences demonstrated to be important in heavy-metal regulation (3, 18). Mice which developed from these injected eggs were fed zinc water and made anemic with phenylhydrazine as before. RNA was extracted from various tissues of the transgenic mice and assayed for hβG RNA by hybridization to an hβG oligonucleotide as described above. In these transgenic mice lacking the MThGH gene, high levels of hβG

TABLE 1. Quantitation of hβG, MThGH, and MT-I RNA in MThGH-hβG transgenic mice[a]

Sample source	Quantitation (molecules/cell) in mouse:											
	21-5			21-7			21-8			21-9		
	hβG	MThGH	MT-I	hβG	MThGH	MT-I	hβG	MThGH	MT-I	hβG	MThGH	MT-I
Blood	0		0	0			0		0	0	0	0
Spleen	0	0	15	0	0	7	0	0	29	0	0	26
Liver	11	168	1,487	11	148	1,338	0	26	1,920	17	16	1,911
Kidney	3	151	250	2	30	351	1	68	455	0	4	380
Brain	0	0	144	0	1	115	1	32	140	6	5	183
Intestine	75	1,960	1,030	75	3,860	830	8	58	1,030	75	2,277	2,650
Heart	1	4	196	1	8	93	1	26	112	0	0	150

[a] hβG RNA was quantitated by S1 nuclease assay with the 3' probe illustrated in Fig. 2; the intensity of the 85-base band in each sample was compared with the same band in the hβG RNA controls. Several of these values were subsequently confirmed by solution hybridization with an oligonucleotide probe (see the text). MThGH and MT-I RNA levels were determined as described by Palmiter et al. (15). The ratio of liver/intestine MT-I mRNA in the zinc-fed animals described here is lower than the liver/intestine ratio in cadmium-injected animals described in reference 15. The differences reflect the route of administration of heavy metals. Mice 21-5, 21-7, 21-8, and 21-9 contained 1, 1, 10, and 1 copies per cell, respectively, of the MThGH-hβG construct.



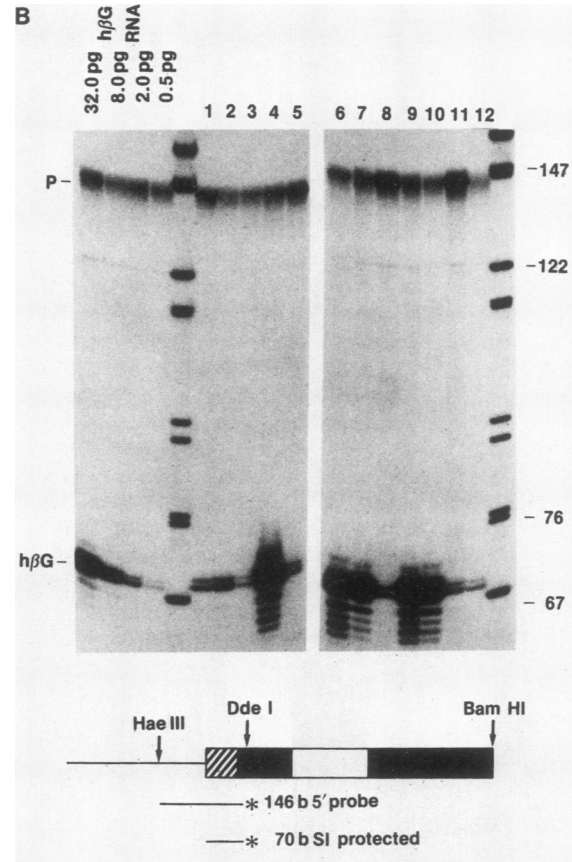


FIG. 3. S1 nuclease assay of the 3′ and 5′ ends of intestinal RNA from all 11 MThGH-hβG transgenic mice. Total intestinal RNA (25 μg) from MThGH-hβG transgenic mice was hybridized to the 3′-end-labeled (A) or 5′-end-labeled (B) strand-separated probes illustrated below the figures. S1-protected DNA then was analyzed on an 8.0% urea–polyacrylamide gel. Lanes 1 to 12 of panels A and B represent analysis of the same RNAs with the different probes. Lanes: 1, mouse 23-8 (negative control), 2, 21-8; 3, 23-4; 4, 21-9; 5, 21-3; 6, 21-5; 7, 21-4; 8, 22-6; 9, 21-7; 10, 23-2; 11, 23-5; 12, 23-9. Lane 13 in panel A is an additional negative control. The weak bands detected in the negative control RNA in panel B are due to a small amount of cross-hybridization between the 5′ probe and mβG mRNA which is present in intestine as a result of blood contamination (Table 2). No cross-hybridization occurs with the 3′ probe.

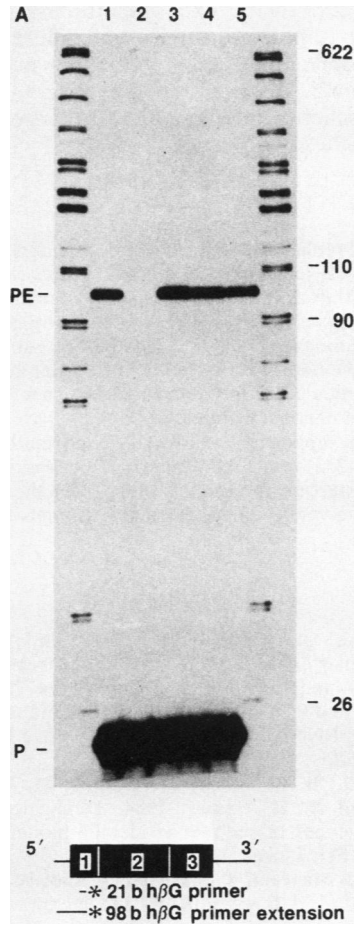
RNA were detected in reticulocytes and spleen (Table 2) and lower levels were detected in several other tissues.

To determine whether the hβG mRNA detected in nonerythroid tissues reflects contamination by blood cells, we also quantitated the amount of mβG RNA. All of the tissues of each mouse contained an essentially constant proportion of hβG and mβG mRNA (Table 2). Therefore, it appears that the hβG RNA detected in nonerythroid tissues is due to blood contamination and that the human globin gene in these mice is expressed in an erythroid-specific manner. Moreover, the genes appear to be expressed at a high level. The amount of hβG mRNA was 45 to 88% of mβG mRNA (Table 2). These values are equivalent to 900 to 1,600 molecules per cell of hβG RNA.

To determine whether transcription of the hβG gene in these mice is initiated correctly, we mapped the 5′ ends of reticulocyte RNA by primer extension. The data (Fig. 4A) illustrate that the hβG RNA in these three transgenic mice had the same 5′ end as RNA extracted from human reticulocytes. Therefore, transcription appears to be initiated at the authentic start site. The ratio of hβG to mβG RNA in the reticulocytes of these mice also was determined by primer extension. The hβG and mβG oligomers were 5′ end labeled together, hybridized together with blood RNA from mice 35-4, 35-5, and 36-3, and extended with reverse transcriptase. Because the 5′ untranslated region of mβG mRNA is 2 bases longer than hβG mRNA, the primer-extended products can be separated on a denaturing acrylamide gel. Mouse 35-4 contains approximately equal amounts to hβG and mβG RNA, and mice 35-5 and 36-3 contain only slightly less hβG RNA than mβG RNA (Fig. 3B). These results confirm the data for blood RNA presented in Table 2. The hβG RNAs in these mice not only have the correct 5′ ends but must also be correctly spliced and terminated because authentic hβG polypeptides are easily detected in lysates of circulating erythrocytes on isoelectric focusing gels (data not shown).

DISCUSSION

In experiments to be published elsewhere, we showed that hβG genes devoid of any heterologous sequences can be expressed in transgenic mice in an erythroid-specific manner at levels approaching that of endogenous β-globin genes.



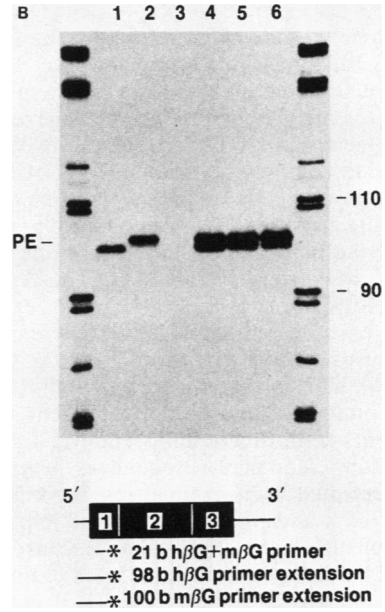


FIG. 4. Primer extension analysis of the 5′ ends of hβG and mβG RNAs in reticulocytes of transgenic mice. (A) Total reticulocyte RNA was hybridized to the 5′-end-labeled human β 21-mer, and the primer was subsequently extended with reverse transcriptase and analyzed on an 8% urea–polyacrylamide gel. P, position of the 21-base primer; PE, primer-extended product. The markers are *Hpa*II-cut pBR322. Lanes: 1, human reticulocyte RNA (~500 pg of hβG RNA); 2, control mouse reticulocyte RNA (0.5 μg); lanes 3 to 5, 0.5 μg of reticulocyte RNA from mice 35-4, 35-7, and 36-3, respectively. (B) Total reticulocyte RNAs were hybridized to equimolar amounts (~0.05 pmol each) of the human and mouse β oligonucleotides which had been 5′ end labeled in the same reaction mixture. The primers then were extended with reverse transcriptase and analyzed on 5% urea–polyacrylamide gel. Lanes: 1, human reticulocyte RNA; 2, mouse reticulocyte RNA; 3, tRNA; 4 to 6, reticulocyte RNA from mice 35-4, 35-5, and 36-3, respectively; PE, primer-extended product. Schematic representations of globin mRNA are illustrated below panels A and B. Thin lines mark 5′- and 3′-untranslated regions, and boxes represent exons. 5′-labeled oligonucleotide primers and primer-extended products are illustrated below the mRNA. The 5′ untranslated region of mβG mRNA is 2 bp larger than hβG mRNA; therefore, the primer-extended products can be separated on denaturing polyacrylamide gels.

hβG gene expression in those mice resembles that described here for the transgenic mice bearing the *Sst*I-*Kpn*I fragment; thus, the metallothionein sequences remaining in the *Sst*I-*Kpn*I fragment apparently do not influence hβG gene expression. The conclusion from both of these studies is that DNA sequences associated with the hβG gene direct its expression to erythroid cells. The location of these sequences is currently unknown; however, our results and those obtained by transfection into tissue culture cells suggest that sequences lying both 5′ and 3′ of the *Nco*I site at +53 are involved (5, 23). A current hypothesis suggests that these sequences bind proteins that may only be produced in cells of the erythroid lineage and that binding these proteins is instrumental in

TABLE 2. Quantitation of hβG and mβG RNA in hβG transgenic mice[a]

Sample source	Quantitation (pg of mRNA/μg of total cellular RNA) in mouse:								
	35-4			35-5			36-3		
	hβG	mβG	hβG/mβG	hβG	mβG	hβG/mβG	hβG	hβG	hβG/mβG
Blood	1,245	1,410	0.88	839	1,370	0.61	690	1,520	0.45
Spleen	234	273	0.86	194	264	0.73	132	454	0.29
Liver	16	22	0.73	14	27	0.52	11	35	0.31
Kidney	16	15	1.06	27	48	0.56	16	35	0.46
Brain	25	25	1.00	12	32	0.38	16	34	0.47
Intestine	41	44	0.93	33	57	0.58	3	10	0.30
Heart	63	53	1.20	85	123	0.69	50	126	0.40

[a] Blood cell values can be converted to molecules per cell by multiplying by 1.3. Mice 35-4, 35-5, and 36-3 contained 50, 100, and 5 copies per cell, respectively, of the *Sst*I-*Kpn*Ifragment (see thin, hatched box in Fig. 1).

committing the hβG gene to be expressed. DNA sequences with these properties are often referred to as tissue-specific enhancers (1, 10, 16, 21).

The most remarkable finding described here is the strong influence of the neighboring MThGH gene on hβG gene expression. One might have expected the two transcription units to function completely independently of each other or possibly in a codominant manner, with each gene being expressed in the appropriate tissues but also in inappropriate tissues under the influence of the neighboring transcription unit. Instead, we observed that the MThGH transcription unit completely suppressed hβG gene expression in erythroid tissues and redirected hβG gene expression in a fashion similar to the MThGH gene (Table 1). One interpretation of these data is that sequences within the MThGH transcription unit or flanking DNA prevent the hβG enhancer elements from functioning. The MT-I promoter and metal regulatory sequences, sequences within the hGH gene, or the residual vector sequences (a combined total of 1.5 kilobases of λ and pBR322) might be implicated in the suppression of hβG gene expression. Because no MThGH mRNA could be detected in reticulocytes of these zinc- and phenylhydrazine-treated mice (Table 1), it is unlikely that transcription of the MThGH gene directly interferes with hβG gene expression in erythroid cells. We noted inhibitory effects of procaryotic vector sequences on β-globin gene expression (T. M. Townes et al., manuscript in preparation), and although the procaryotic sequences tested previously are different from those present in this construct, the hβG gene may be extremely sensitive to the inhibitory effects of any procaryotic DNA. In fact, procaryotic vector sequences were included in the β-globin constructs injected previously (8, 12, 19, 20) and may be responsible for lack of expression.

A curious feature of the inhibitory sequences is that they abolish hβG gene expression in erythroid cells but allow expression in other cells; the pattern of hβG in other tissues resembles that of the neighboring MThGH gene (Table 1). Considering that the hGH gene is not expressed in transgenic mice on its own (11), it seems reasonable to implicate the MT-I promoter in directing the transcription of both the MThGH and neighboring hβG genes. Expression of hβG genes in the intestine, a prime site of MT-I synthesis in zinc-fed mice, is certainly consistent with this observation. The hβG mRNAs are initiated and terminated properly (Fig. 3A and B); thus, the data suggest that the MT-I sequences that lie between the *Bgl*II (+64) and *Sst*I (−150) sites are acting like an enhancer to influence transcription from the hβG promoter located about 500 bp away. We observed a similar enhancerlike effect of MT-I sequences on simian virus 40 early gene expression when the simian virus 40 enhancers were removed (R. D. Palmiter et al., submitted for publication). In that case, we were able to show that simian virus 40 early gene expression became metal inducible, clearly implicating the metal regulatory elements that lie within the MT-I gene promoter. We also observed that MThGH gene expression is not identical to the endogenous MT-I gene (the relatively low level of expression in the liver is particularly obvious, especially since high levels of MThGH expression are observed in the livers of transgenic mice containing the MThGH gene alone [15]); thus, it is conceivable that the adjacent hβG gene also influences MThGH gene expression in an unexpected manner.

Genes in higher eucaryotes tend to be widely spaced; usually many kilobases of DNA with no known function lie between them. Perhaps this spacer DNA plays an important role in isolating the various transcription units from neighboring influences. In the experiments described here, we juxtaposed two transcription units with different tissue specificities, developmental timing, and responsiveness to environmental stimuli. Perhaps we are observing the consequences of conflicting interactions of enhancerlike elements that lie within these two genes.

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