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Regulated Expression of Human γ -, β -, and Hybrid $\gamma\beta$ -Globin Genes in Transgenic Mice: Manipulation of the Developmental Expression Patterns

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Summary

We have introduced the human fetal γ - and adult β -globin genes into the germ line of mice. Analysis of the resulting transgenic mice shows that the human γ -globin gene is expressed like an embryonic mouse globin gene; the human β -globin gene is expressed (as previously shown) like an adult mouse globin gene. These results imply that the regulatory signals for tissue- and developmental stage-specific expression of the globin genes have been conserved between man and mouse but that the timing of the signals has changed. Because the two genes are expressed differently, we introduced a hybrid $\gamma\beta$ -globin gene construct. The combination of the regulatory sequences resulted in the expression of the hybrid gene at all stages in all the murine erythroid tissues.

Introduction

The murine β -like globin locus comprises seven genes closely linked within 65 kb of DNA (Jahn et al., 1980; Leder et al., 1980), organized as follows: 5'- γ - β h0- β h1- β h2- β h3- β ^{maj}- β ^{min}-3'. In normal mouse development primitive nucleated erythrocytes formed in the yolk sac blood islands are released into circulation at about day 9.5 of gestation. These embryonic cells persist in circulation until about day 16.5 of gestation (Kovach et al., 1967) and contain the embryonic globin chains γ and z (Fantoni et al., 1967). These chains are encoded by the genes γ and β h1, respectively (Hansen et al., 1982; Farace et al., 1984). The mouse fetal liver becomes the major site of erythrocyte formation shortly after day 10.5 of gestation and remains a site of erythropoiesis almost to birth (Rifkind et al., 1969). The liver-generated non-nucleated erythrocytes are released for the first time into circulation on day 12.5 of gestation and, in contrast to erythrocytes in man, contain only adult globin chains encoded by the adult β ^{maj} and β ^{min} genes (Konkel et al., 1979). At about day 16.5 of gestation, synthesis of the same adult globin chains begins in the spleen and bone marrow, which remain the sites of erythropoiesis in adult life (Borghese, 1959).

The human β -like globin genes are a cluster of five active genes in the order 5'- ϵ - $G\gamma$ - $A\gamma$ - δ - β -3' occupying approximately 60 kb of DNA. During development the different genes are expressed in different tissues and at different times, i. e., the embryonic ϵ gene is expressed in the yolk sac, the fetal $G\gamma$ and $A\gamma$ genes are expressed primarily in the fetal liver, and the adult δ and β genes are

expressed primarily in bone marrow (reviewed by Maniatis et al., 1981). Humans, as opposed to most species, including mice (see above), have undergone a process of fetal recruitment resulting in a separate set of fetal genes. This has also been observed in other primates, in goats, and in sheep (Schon et al., 1981; Kretschmer et al., 1981).

In the past few years considerable progress has been made in our understanding of the DNA sequences necessary for the transcription of the globin genes (Dierks et al., 1983; Grosveld et al., 1982). More recently, by DNA-mediated gene transfer into cultured mouse erythroleukemia (MEL) cells, it has been shown that expression of the human β -globin gene, but not the ϵ - and γ -globin genes, is regulated during MEL cell differentiation (Wright et al., 1983; Chao et al., 1983; Spandidos and Paul, 1982). The results with hybrid genes suggest that the DNA sequences that regulate β -globin gene expression during MEL cell differentiation are located both 5' and 3' of the translation initiation site (Wright et al., 1984; Charnay et al., 1984). Although very useful, the cell systems simulate in vivo erythropoiesis only in part, and they therefore have certain disadvantages, in particular, for studying the switch from embryonic to fetal to adult globin gene expression.

A more powerful approach to the study of switching mechanisms has been the development of methods to introduce foreign genes successfully into the germ line of mice (Gordon et al., 1980). A number of genes that have been introduced into mice by microinjection of fertilized eggs have shown, when expressed, the same regulation characteristics as their endogenous counterparts (Brinster et al., 1981; Palmiter et al., 1982; Grosschedl et al., 1984; Swift et al., 1984; Hanahan, 1985; Magram et al., 1985; Adams et al., 1985). To study the molecular mechanisms involved in the developmental regulation of globin gene expression, we introduced the human adult β -globin and fetal γ -globin genes into the germ line of mice. We show that in the transgenic mouse system the human β -globin gene is regulated as an adult globin gene (see also Magram et al., 1985; Townes et al., 1985), but the human fetal $A\gamma$ -globin gene is regulated as an embryonic globin gene. To show whether the multiple regulatory sequences that have been identified in cell culture systems are required for this complete developmental process, we have also introduced a recombinant globin gene construct containing the 5' part of the human $A\gamma$ gene linked to the 3' part of the human β -globin gene. The results show that this recombinant gene is correctly transcribed both in the embryonic and the adult stage of mouse development.

Results

Expression of Human $A\gamma$ -Globin and β -Globin mRNA in Mouse Embryos

A 3.3 kb HindIII fragment containing the entire human $A\gamma$ -globin gene in addition to 1.3 kb of 5'-flanking DNA and

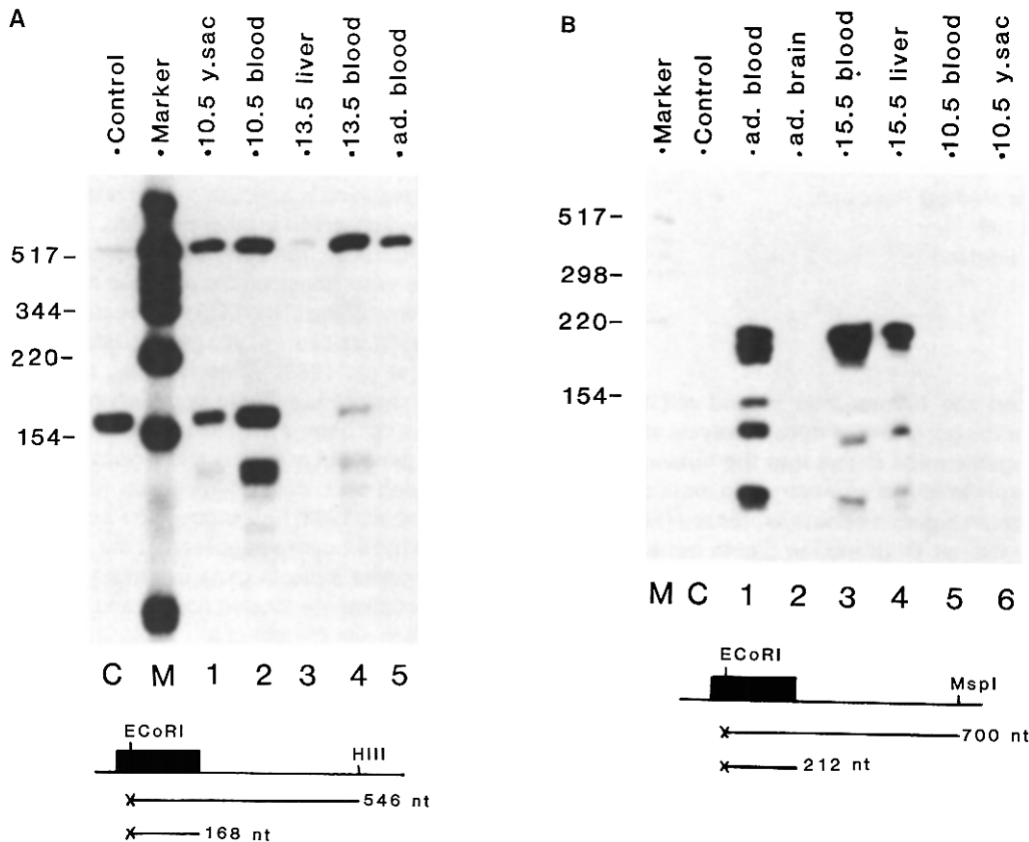


Figure 1. Expression of Human γ - and β -Globin Genes in Transgenic Mouse Tissues

(A) Expression of the human γ -globin gene in transgenic mouse tissues. Ninety-one eggs survived the microinjection and were transferred into the oviducts of pseudopregnant recipients; 19 mice were born. Seven transgenic pups were identified by Southern blot hybridization of DNA isolated from tail biopsies and were found to carry between two and 50 copies of the γ -globin gene. Shown is the expression pattern of line γ 18.

Total RNA was extracted from the following tissues (see Experimental Procedures): day 10.5 embryonic yolk sac and blood, day 13.5 fetal liver and blood, and adult blood. Thirty micrograms of this RNA was hybridized at 52°C to a 3' end-labeled probe derived from the 3' end of the human γ -globin gene (see diagram). Seven micrograms of RNA from an γ -globin-producing cell line (PUTKO; Klein et al., 1980; Rutherford et al., 1981) was used as a positive control. Normal transcription termination of the γ -globin gene produces an mRNA that protects 168 nucleotides of a 3' end-labeled EcoRI-HindIII fragment from S1 digestion. The shorter fragment in the positive lanes is a variable S1 nuclease analysis artifact (Wright et al., 1983). Autoradiography was for 16 hr at -70°C with an intensifying screen. Sizes (at left) were estimated with an end-labeled HindIII digest of pBR322.

(B) Expression of the human β -globin gene in transgenic mouse tissues. Ten mice were born after β -globin gene microinjection and transfer of eggs to oviducts. Two of these carried the complete β -globin gene. The mouse carrying five copies of the gene was bred to homozygosity. Shown is the β -globin gene expression pattern of line β 7.

Total RNA was extracted from different tissues of mice carrying the human β -globin gene; 30 μ g of this RNA was hybridized at 52°C to a 3' end-labeled probe derived from the 3' end of the human β -globin gene (see diagram). One nanogram of sickle-cell RNA was used as a positive control, which was visible only after long exposures. Normal transcription termination of the human β -globin gene promoter produces an mRNA that protects a 212 nucleotide probe fragment from S1 digestion (Wright et al., 1983). The different lanes represent mRNA from adult mouse blood and brain, day 15.5 fetal blood and liver, and day 10.5 embryonic blood and yolk sac. Autoradiography was for 1/2 hr at -70°C with an intensifying screen. Sizes were estimated with an end-labeled HindIII digest of pBR322 DNA.

370 nucleotides of 3'-flanking DNA was microinjected preferably into the male pronucleus of F2 (CBA \times C57Bl/10) zygotes (see legend, Figure 1). Of the seven transgenic lines obtained, one line was a high-copy chimeric line and another was very slow breeding. Therefore, these two lines were not included in this study. Two of the five remaining lines were found to express the human γ -globin gene in the same developmental stage, although at different levels (data not shown).

Figure 1 shows the expression pattern of line γ 18 by S1 nuclease protection assays on RNA samples isolated

from different transgenic tissues throughout development (see Experimental Procedures). The RNA samples were initially assayed for the presence of human γ -globin mRNA by a probe derived from the 3' end of the human γ -globin gene (Figure 1A). A 168 nucleotide fragment is protected from S1 nuclease digestion by the correctly terminated human fetal γ -globin mRNA. As shown in Figure 1A, at 10.5 days of development both the embryonic yolk sac (lane 1) and blood (lane 2) RNA samples protect a 168 nucleotide fragment corresponding to the correct γ -globin mRNA 3' end, as well as some smaller frag-

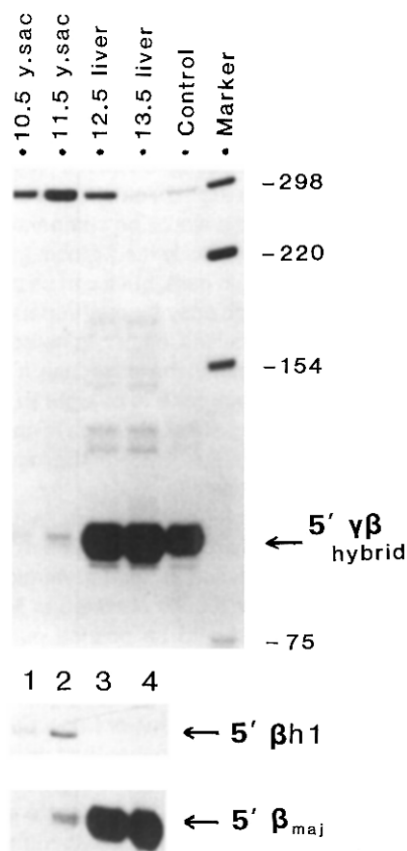


Figure 2. Expression of a Human Hybrid 5'- γ - β -3'-Globin Gene in Transgenic Mouse Tissues

One hundred seventy-two eggs survived the microinjection and were transferred into pseudopregnant recipients; 25 mice were born. Seven of these were found to be transgenic, and they carried between 1–100 copies of the hybrid gene. One line was a very slow breeder and therefore was excluded from this study. Out of the remaining six, three were found to express the human hybrid globin gene. Shown is the expression pattern obtained with mouse $\gamma\beta 11$.

Total RNA was extracted from the following tissues (see Experimental Procedures): day 10.5 and 11.5 yolk sac and day 12.5 and 13.5 liver. Ten micrograms of this RNA was hybridized at 52°C to a 300 nucleotide BglII–ApaI 5' end-labeled probe derived from the 5' end of the human $\Lambda\gamma$ -globin gene. Normal transcription from the $\Lambda\gamma$ -globin gene promoter produces an mRNA that protects a probe fragment of 90 nucleotides from S1 digestion. Seven micrograms of PUTKO cell RNA (see Figure 1) was used as a positive control (Klein et al., 1980; Rutherford et al., 1981). Lanes 1, 2 and 3, 4 were joined from the same autoradiograph to facilitate presentation of the results.

The two insets at the bottom show the autoradiographic signals obtained from S1 nuclease protection assays of probes specific for $\beta h 1$ and β^{maj} mRNAs using 1 μ g of total RNA. The $\beta h 1$ probe was a 255 nucleotide HinfI fragment derived from the third exon of the $\beta h 1$ gene, and the β^{maj} probe was a 700 nucleotide HindIII–NcoI fragment derived from the second exon of the β^{maj} gene. Specific activities of these two probes and time of exposure of the autoradiograph were equalized to allow a comparison of the ratio of the endogenous embryonic to fetal globin signal. Sizes were estimated from an end-labeled HinfI digest of pBR322 DNA.

ments that represent a known S1 artifact (e. g., Wright et al., 1983). In contrast, expression of the $\Lambda\gamma$ gene is shut off in day 13.5 liver (Figure 1A, lane 3), as found for the embryonic mouse genes (see below). Just as is the case for the mouse embryonic mRNA, the human γ -globin mRNA

is still present in day 13.5 blood (Figure 1A, lane 4), albeit in decreased amounts in comparison with day 10.5 blood. This leftover signal represents the decreasing amount of embryonic yolk sac-derived erythrocytes that continue to be present until day 16.5 of development (Kovach et al., 1967). No $\Lambda\gamma$ -globin mRNA can be detected in adult blood (Figure 1A, lane 5) or nonerythropoietic tissues of any developmental stage (data not shown). A 5' S1 analysis of the same RNA samples also showed correct initiation of transcription (not shown, but see below).

These results show that the fetal human globin gene behaves as an embryonic mouse gene in the mouse developmental cycle, as might be predicted from previous studies (Czelusniak et al., 1982; Hill et al., 1984; Hardies et al., 1984). After fetal recruitment the human γ genes may be subjected to a shift in the timing of the appearance of *trans*-acting signal(s), or, alternatively, an alteration may occur in the *cis*-acting sequences and/or factor(s) in fetal erythroid cells. Our results indicate that the mouse embryonic factor(s) still act on the human γ gene sequences and correctly regulate expression of the human $\Lambda\gamma$ gene as an embryonic gene. Hence, we favor the first model. Our results also demonstrate that the 3.3 kb HindIII $\Lambda\gamma$ -globin gene fragment introduced into the mouse system contains sufficient information to drive transcription in a tissue- and developmental stage-specific manner.

To determine the pattern of expression of a foreign human β -globin gene and subsequently use it as a control for our hybrid globin gene experiments (see below), we also introduced a 5' HpaI–3' ClaI human DNA fragment containing the adult human β -globin gene with 0.8 kb of 5'-flanking DNA and 2.5 kb of 3'-flanking DNA into the germ line of transgenic mice. Figure 1B shows an S1 nuclease protection assay on RNA samples isolated from erythroid and nonerythroid transgenic mouse tissues at different stages of development. At 10.5 days neither yolk sac RNA (Figure 1B, lane 6) nor blood RNA (Figure 1B, lane 5) shows a positive protection signal. At 15.5 days of development human β -globin mRNA is abundant in fetal liver (Figure 1B, lane 4) and fetal blood (Figure 1B, lane 3). In the adult stage, human β -globin mRNA continues to accumulate in blood (Figure 1B, lane 1), but in brain tissue the signal is not detectable (Figure 1B, lane 2). These experiments confirm earlier studies (Magram et al., 1985; Townes et al., 1985) showing that transgenic mice carrying the human adult β -globin gene can express the gene correctly in a tissue-specific and developmentally regulated manner.

Because different expression patterns for the human fetal and adult globin genes in transgenic mice are observed, this experimental system offers the possibility of defining the DNA sequences involved in developmental regulation by using human γ -globin, β -globin hybrid gene constructs.

Expression of a Hybrid 5'- γ - β -3' Gene Throughout Mouse Development

To begin defining the DNA sequences involved in the regulated expression of either the human $\Lambda\gamma$ - or γ -globin

genes, we microinjected a hybrid gene construct into mouse fertilized eggs. The 5' part of the human $\Lambda\gamma$ -globin gene (up to the BamHI site of the second exon) was adjoined to the complementary 3' part of the human β -globin gene. This construct was chosen on the basis of K562 and MEL cell experiments showing that (at least) the 5' half of the γ -globin gene contains sequences responsible for its expression in embryonic/fetal cell types (Kioussis et al., 1985; Ley and Bodine, personal communication) and that the 3' end (in addition to the 5' end) of the human β -globin gene contains regulatory sequences involved in its expression in adult cells (Wright et al., 1984; Charnay et al., 1984).

The 5'-HindIII- $\Lambda\gamma$ -BamHI- β -ClaI-3' hybrid gene, containing the same 5' sequences as in the $\Lambda\gamma$ -globin gene fragment and the same 3'-flanking sequences as in the β -globin gene fragment, was microinjected into mouse zygotes (see Experimental Procedures and Figure 2). Three mice expressed the human hybrid globin gene at the same developmental stages in a tissue-specific manner, although the total level of RNA varied between mice (data not shown).

Figure 2 shows the expression pattern of line $\gamma\beta 11$ (carrying approximately five copies of the foreign DNA) as determined by 5'-end S1 nuclease protection assays on RNA samples isolated from different transgenic tissues throughout development. Human globin mRNA levels were determined in samples from day 10.5 and 11.5 yolk sacs (Figure 2, lanes 1 and 2) and day 12.5 and 13.5 livers (Figure 2, lanes 3 and 4). The 90 nucleotide protected fragment represents correct initiation of transcription from the hybrid gene (Figure 2, control lane). Globin-specific RNA synthesis is restricted to the two hemopoietic tissues of the developing mouse embryo, and RNA continues to accumulate in adult blood (data not shown). To confirm that the ratio between embryonic yolk sac and fetal liver mRNA levels for the hybrid $\gamma\beta$ -globin gene is the same as the normal ratio between the endogenous mouse genes, we performed a quantitative S1 nuclease analysis of the same RNA samples with probes of the same specific radioactivity for endogenous $\beta\text{h}1$ and β^{maj} transcripts. The insets in Figure 2 show that a similar ratio of embryonic yolk sac globin mRNA to fetal liver globin mRNA is found, although it should be noted that the overall levels of expression for the foreign gene are 10-fold lower than for the endogenous globin genes in this particular mouse. The low-intensity protected band in the day 11.5 yolk sac RNA sample probed with the 5' β^{maj} probe (Figure 2, lane 2, bottom) may represent either early-circulating, non-nucleated erythrocytes of liver origin in the yolk sac sample or a very low level of transcription of the β^{maj} -globin gene in the yolk sac. The latter possibility would be similar to very recent evidence that shows a low level of transcription of the human γ -globin gene in human yolk sac (Peschle et al., 1985).

Discussion

Our results clearly show that the manipulation of regulatory elements has resulted in a new developmental ex-

pression pattern for the hybrid gene: there is an embryonic stage due to the 5' half of the γ -globin gene and a fetal/adult stage due to the 3' half of the β -globin gene. This implies that the γ -globin gene that is first activated in the mouse is not (irreversibly) inactivated in the adult stage, since the factors acting on the 3' β -globin sequences lead to an active expression of the "normally" silent γ -globin promoter. This would be comparable to the human in vivo situation: normally the γ -globin gene is not expressed in adult erythroid cells, but it can be stimulated to express reasonably efficiently by erythropoietic stress or by natural deletion mutations leading to hereditary persistence of fetal hemoglobin. In the latter case, it has been suggested that the γ -globin gene is brought in proximity to a (nonglobin) enhancing element, which is normally located a long distance from the 3' side of the locus (for review, see Collins and Weissman, 1984).

The fact that the human β -globin gene contains a 3' element, which can positively regulate a (γ -globin) promoter that is normally not expressed in adult erythroid cells, is in agreement with similar results obtained in MEL cells (Wright et al., 1984). It should be pointed out that the γ -globin gene alone is transcribed in MEL cells and that the addition of the 3' β -globin gene only regulates its expression, i. e., causes a stimulation of transcription over a basal level after differentiation rather than an activation from zero. In the case of the γ -globin gene, this discrepancy is probably due to a bypass of some activation control mechanism in the MEL cell experiments that results from the transfection/dominant marker selection method used to ensure integration of the newly introduced DNA into an actively expressing chromatin structure (Smithies et al., 1985). Experiments with the γ -, and β -, and $\gamma\beta$ -globin transgenes in mice are in complete agreement with transfection experiments in K562 cells. In those cells an introduced γ -globin gene is correctly expressed even when only the 5' half of the gene is present, although an introduced β -globin gene is not expressed (Kioussis et al., 1985). The lack of β -globin gene expression is solely caused by 5'-end sequences, as determined by hybrid gene transfections. In contrast 3'-end β -globin sequences have no influence on an active or inactive promoter in K562 cells (Kioussis et al., 1985; Antoniou et al., unpublished data). Together these results suggest that in the simplest model the 5' sequences might be involved in an on/off, positive/negative regulation of the gene (possibly by factors such as reported by Emerson et al. [1984]), whereas the 3' sequences might act only on an already activated (but not necessarily expressing) gene. For example, the absence or presence of some *trans*-acting factor(s) might act on the 5' sequences to lead to the first (low level of) expression of the β -globin gene in human fetal liver in vivo. Additional factors or a changed balance of factors in a later stage would interact with the 5' and 3' sequences to give fully active expression. Of course, more complicated models are possible in which the multiple components only specifically interact with each other to yield regulated expression.

It should be noted that only a fraction (30%–50%) of the transgenic mice express the foreign globin gene, and they

do so at extremely variable levels (Magram et al., 1985; Townes et al., 1985; and our results). This has also been seen in other systems, with the exception of the α -fetoprotein gene when it is introduced with a large 5'-flanking sequence (Krumlauf and Tilghman, personal communication). This variable expression is attributed to a position effect, i. e., a dependence on the activity of the site of integration of the foreign gene in the host genome. This actually implies that yet another level of control is not properly exerted and that some regulatory sequences are absent from the newly introduced DNA. In the case of the globin genes, these could be some sort of multigene locus-activating sequences, clearly different from and dominant over the tissue- and developmental stage-specific sequences, which are directly in *cis* to the globin genes as described above. The absence of such sequences would lead to position effects such as those observed in vivo for particular thalassemias (Kioussis et al., 1983) or in transgenic mice as described above. Possible candidates for such sequences might be the extreme DNAase I-hypersensitive sites far upstream of the ϵ -globin gene and far downstream of the β -globin gene, which could define an erythroid-active domain (Tuan et al., 1985).

Experimental Procedures

Microinjection and Animal Treatments

F2 hybrid fertilized eggs of CBA \times C57Bl/10 parents were isolated, and pronuclei were injected with approximately 2 pl of a solution containing approximately 2 μ g/ml of DNA fragments in TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA). The eggs that survived the microinjection were incubated overnight in culture medium. The next morning, only the two-cell stage embryos were transferred into the oviducts of recipient pseudopregnant females (for review, see Brinster et al., 1985). After birth and weaning, the pups were analyzed for the presence of the injected DNA by Southern blot analysis of tail genomic DNA (Southern, 1975).

To obtain embryonic tissues for expression analysis, appropriate matings were set up. The day the mating plug was observed was designated day 0.5, and embryos at the appropriate stage of development were dissected and washed in phosphate-buffered saline (PBS) containing 10 U/ml heparin to remove maternal blood contamination. Transgenic embryos in each litter were determined by placenta dot blot analysis (e. g., Brinster et al., 1985). Total RNA was prepared from the dissected tissues that proved to carry the foreign globin genes (see below). In some cases mice were made anemic by three injections (every 12 hr) of a 0.4% phenylhydrazine solution (Cheng et al., 1974) at a dose of 0.2 ml per 25 g of bodyweight.

Purification of RNA from Mouse Tissues

Whole tissues were washed in PBS, and cells were lysed by homogenization in 6 M guanidinium chloride (Chirgwin et al., 1979). The homogenate was phenol-extracted; nucleic acids were ethanol-precipitated, dissolved in 10 mM Tris (pH 7.5), 10 mM MgCl₂, 50 mM NaCl, and treated with RNAase-free DNAase. RNA was phenol-extracted and ethanol-precipitated. Concentrations were determined from the absorbance at 260 nm and confirmed by electrophoresis on 2% agarose gels; the latter also showed that the RNA preparations were undegraded.

S1 Nuclease Analysis

Globin-specific mRNA was assayed by S1 nuclease analysis (Berk and Sharp, 1977; Weaver and Weissmann, 1979). Probes were end-labeled by either T4 polynucleotide kinase or reverse transcriptase, and specific activities were estimated by Cerenkov counting. The labeled probe was hybridized to 20–50 μ g of total tissue RNA in 10–15 μ l of 40 mM Pipes (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% (recrystallized) formamide for about 16 hr at 52°C. After hybridization, samples

were digested for two hr at 20°C with 3000 U of S1 nuclease in 300 μ l of 200 mM NaCl, 30 mM NaOAc (pH 4.8), 2 mM ZnSO₄. The DNA protected from S1 digestion was ethanol-precipitated and then electrophoresed on 6% urea-polyacrylamide gels.

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