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Analysis of the human α-globin gene cluster in transgenic mice

J. A. SHARPE*, D. J. WELLS†, E. WHITELAW‡, P. VYAS*, D. R. HIGGS*, AND W. G. WOOD*§

*Medical Research Council Molecular Haematology Unit, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, United Kingdom; †Department of Veterinary Basic Sciences, Royal Veterinary College, Royal College Street, London, NW1 OTU, United Kingdom; and ‡Biochemistry Department, University of Sydney, Sydney, 2006 Australia

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ABSTRACT **A 350-bp segment of DNA associated with an erythroid-specific DNase I-hypersensitive site (HS −40), upstream of the α-globin gene cluster, has been identified as the major tissue-specific regulator of the α-globin genes. However, this element does not direct copy number-dependent or developmentally stable expression of the human genes in transgenic mice. To determine whether additional upstream hypersensitive sites could provide more complete regulation of α gene expression we have studied 17 lines of transgenic mice bearing various DNA fragments containing HSs −33, −10, −8, and −4, in addition to HS −40. Position-independent, high-level expression of the human ζ- and α-globin genes was consistently observed in embryonic erythroid cells. However, the additional HSs did not confer copy-number dependence, alter the level of expression, or prevent the variable down-regulation of expression in adults. These results suggest that the region upstream of the human α-globin genes is not equivalent to that upstream of the β locus and that although the two clusters are coordinately expressed, there may be differences in their regulation.**

The α-globin gene cluster lies near the tip of chromosome 16 and consists of three functional genes arranged 5′-ζ2–α2–α1-3′ (1), while the coordinately expressed β-globin gene cluster on the short arm of chromosome 11 includes five functional genes, 5′-ε–$^{G}\gamma$–$^{A}\gamma$–δ–β-3′ (2). Expression of both clusters is regulated in a tissue- and developmental stage-specific manner to produce embryonic ($\zeta_2\varepsilon_2$, $\alpha_2\varepsilon_2$ and $\zeta_2\gamma_2$), fetal ($\alpha_2\gamma_2$), and adult ($\alpha_2\delta_2$ and $\alpha_2\beta_2$) hemoglobins.

Naturally occurring deletions of sequences upstream of either globin gene cluster down-regulate globin gene expression, suggesting that major regulatory sequences lie in these regions (3–8). This was confirmed initially in the β cluster, where the upstream locus control region (β-LCR), marked by the presence of four DNase I-hypersensitive sites (HSs) (9–11), was shown to be essential for high-level, tissue-specific expression of the human β gene in transgenic mice. HSs 2, 3, and 4 are each capable of conferring position-independent expression on a linked β gene, but maximum levels of transcription require the whole β-LCR (12, 13).

A similar pattern of erythroid-specific HSs was identified 4, 8, 10, 33, and 40 kb upstream of the ζ2 mRNA cap site of the α gene cluster (14, 15). However, only the site at −40 kb (HS −40) appeared to have a significant effect on α gene expression (14–16). HS −40 contains binding sites for both erythroid restricted and ubiquitous trans-acting factors (15, 17) with a pattern of protein binding closely resembling that of HS2 of the β-LCR (15, 18).

We have previously shown that expression of human α-globin mRNA in transgenic mice containing HS −40 linked to an α-globin gene is erythroid specific and that high levels are observed irrespective of integration site (14, 16). However, while the β-LCR may confer copy-number dependence on gene expression (9, 19, 20), α-globin gene expression was not dependent on the number of HS −40α copies (14, 16). Furthermore, expression levels were not developmentally stable, being 2- to 4-fold lower in adults than in embryos (16). It may be that the HS −40α construct lacks further cis-acting sequences required for complete regulation of α gene expression. Therefore, we have examined the effect of the additional hypersensitive sites at coordinates −4, −8, −10, and −33 kb on α-globin regulation. Two of the constructs also contained the human embryonic ζ gene, allowing us to determine its developmental pattern of expression relative to the α gene.

MATERIALS AND METHODS

The cosmid constructs cRN24α and cNFGα (21), previously described as fragments 14 and 15 of ref. 14, were linearized with *Cla* I prior to injection. The plasmid constructs HS −40α (15, 16), HS −40$\zeta\alpha$ (22), 4HS$\zeta\alpha$ (23) have been described. For injection into transgenic mice, the DNA fragments were released with *Not* I and purified by gel electrophoresis.

Transgenic mice were produced by microinjection of DNA into the pronuclei of fertilized mouse eggs (24). Transgenic progeny were identified by analysis of tail DNA and were mated to (CBA × C57B6)F_1 mice to establish lines. For analysis of expression in embryonic and fetal mice, transgenic males were mated to (CBA × C57B6)F_1 females; the morning on which a copulatory plug was observed was considered day 0.5 post coitus.

Screening and copy-number analysis of transgenic lines were carried out as described (14). The integrated human DNA was mapped by standard Southern blotting using probes Pst α (25) and RA1.4 (21). Junction fragments and tandem repeat bands were identified after digestion with *Hin*dIII (4HS$\zeta\alpha$ and cNFGα) or *Bam*HI (HS −40α and HS −40$\zeta\alpha$) and hybridization with Pst α or vector (pCV001) probes.

RNA was extracted (26) from individual intact 8.5- to 11.5-day embryos (including yolk sac), from both the blood and fetal liver of individual 12.5- to 18.5-day fetuses and from peripheral blood and spleen of postnatal animals. The RNA probes used in the RNase protection assay (27) were human α-pSP6α132 (28), mouse α-pSPJMαS (28), human ζ-pζ2SP64 (22), and mouse ζ-pSP64Mζ (28).

RESULTS

Two to eight transgenic lines were produced from each of the constructs (Fig. 1; Table 1). The number of copies of the DNA insert ranged from 2 to ≈70, and in at least four lines the founder was a mosaic. From one founder (α37), high- and low-copy-number offspring were obtained, indicating insertion of human DNA into two separate mouse chromosomes. The high- and low-copy-number offspring were bred separately and treated as two independent lines.

By using appropriate probes and restriction enzyme digests, bands characteristic of tandem head to tail repeats

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Abbreviations: LCR, locus control region; HS, hypersensitive site.
§To whom reprint requests should be addressed.

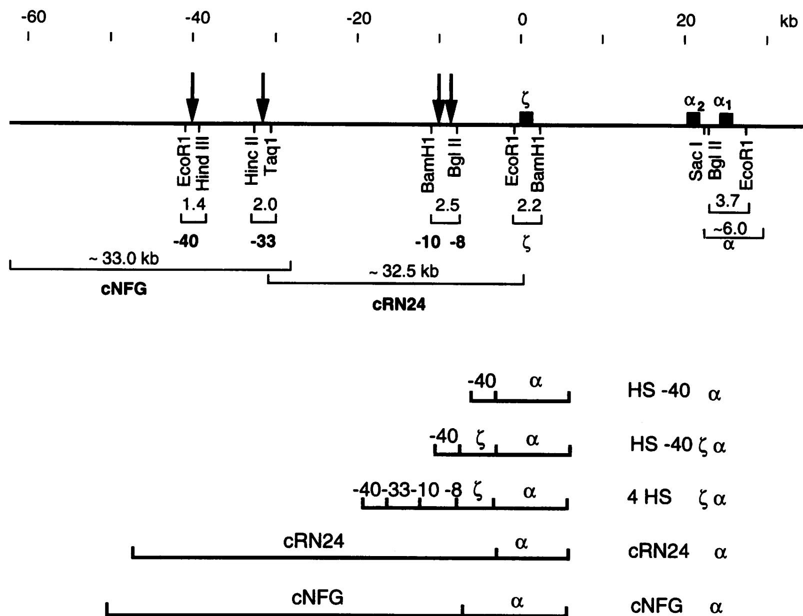

FIG. 1. Map of the human α-globin gene cluster and the constructs used to make transgenic mice. Fragments used to generate the various constructs are shown below the line with their sizes in kilobases; the 3′ end of 6.0-kb α fragment lies in vector sequences within pCL9, from which it was excised.

were observed in all lines. In 5 of 17 lines, more than one junction fragment between the human insert and the mouse genomic DNA were observed. These fragments did not segregate over three generations and presumably reflected separate inserts of human DNA in close proximity within the same chromosome.

Human α-Globin Gene Expression. cRN24α, which contains the HSs −10, −8, and −4, had previously been shown to produce a low level of human α mRNA in one high-copy-number mouse fetus (14). In the three lines bearing this construct, expression was undetectable (<0.05%) in most adult animals and ranged from 0.05% to 1.2% (mean, 0.25%) in embryos. It is clear, therefore, that in the absence of HS −40, little or no α gene activity is observed.

In all lines containing HS −40, human α mRNA was readily detectable in adult blood. The relative levels of human and mouse α mRNA in representative adult animals from each line are shown in Fig. 2 and summarized in Table 1. There is limited variability among animals within each line (2- to 3-fold), but there is considerably greater variation between lines for each of the constructs.

It was previously noted (16) that expression of the human gene from HS −40α appeared to be higher in embryos than in adults, so a complete developmental profile of the human ζ + α mRNA/mouse ζ + α mRNA ratio was carried out on two lines carrying each of the constructs. Embryonic (10.5 days), newborn, and adult samples were examined in all the remaining lines.

Table 1. Copy number and mRNA levels, mean (range)

			Adult		Embryo	
Construct	Line	Copy no.	α^H/α^M mRNA, %	Expression per copy	$(\alpha^H + \zeta^H)/(\alpha^M + \zeta^M)$ mRNA, %	Expression per copy
cRN24α	α48	6–10	0	0	<0.05	
	α47	≈70	0	0	0.05	
	α46	14–18	0	0	0.4 (0.002–1.2)	
cNFGα	α30	3–5	16.8 (11–27)	16.8	16.0 (15–18)	11.1
	α29	4–6	1.0 (0.2–2.4)	0.8	25.0 (21–34)	19.0
	α44	10–14	2.3 (1.4–4.1)	0.8	18.9 (18–21)	4.3
	α42	8–16	5.1 (1.5–7.0)	1.7	17.6 (13–21)	6.2
	α32	16–24	2.2 (0.5–4.7)	0.4	25.2 (22–28)	3.5
	α43	24–36	6.9 (3–10)	0.9	13.2 (11–15)	1.0
	α28	≈60	11.3 (7–16)	0.7	29.0 (16–37)	1.3
	α45	≈70	16.0 (13–21)	0.9	27.8 (21–32)	1.1
4HSζα	α22	2	3.0 (0.6–5.7)	6.0	42.6 (32–52)	59.0
	$\alpha 37_L$	3–5	10.5 (9–16)	10.5	54.0 (32–50)	29.5
	α40	12–20	30.5 (23–35)	7.6	34.8 (27–45)	5.8
	$\alpha 37_H$	20–30	41.4 (27–53)	6.6	42.9 (26–56)	5.2
HS-40ζα	α49	12–20	19.4 (16–23)	4.9	62.0 (56–71)	10.4
	α33	24–36	11.9 (9–15)	1.6	42.2 (22–54)	6.8
HS-40α	α12	8	15.0 (9–22)	7.5	37.0	12.9
	α13	12	13.5 (8–19)	4.4	40.0	9.2
	α14	16	19.0 (11–26)	4.8	—	—
	α11	30	24.9 (23–28)	3.3	42.2	3.9

Expression per copy was calculated as (α^H/human copy no.)/(α^M/4) in adult animals and [($\alpha^H + \zeta^H$)/human copy no.]/[(ζ^M/2) + (α^M/4)] for embryonic data (H, human; M, mouse). Data for HS −40α are taken from ref. 29 for comparison. The mRNA levels in adult animals are the mean of 3–13 animals, 6 weeks of age or older. The data for embryo samples are the mean of 3–15 individual embryos at 10.5 days of gestation.

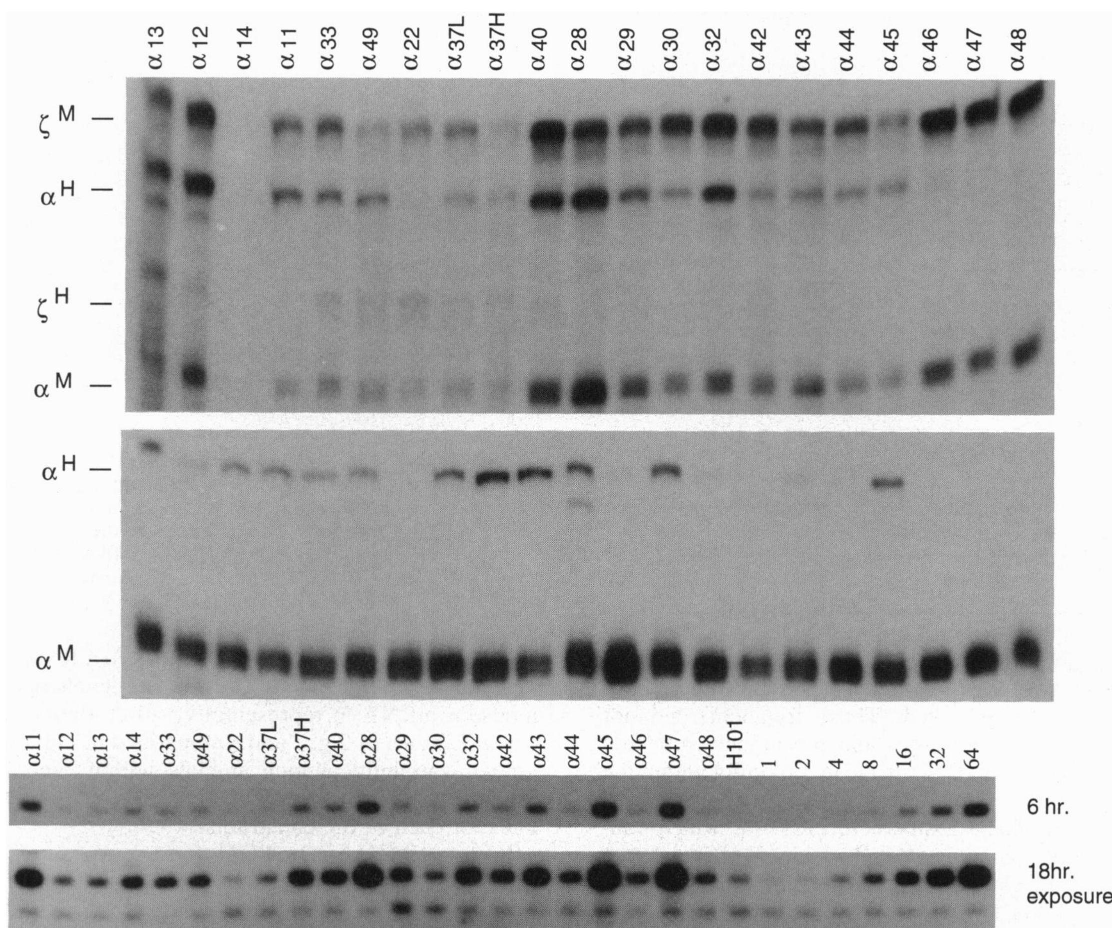

FIG. 2. RNA and DNA analysis of representative transgenic mice from each of the lines. (*Top*) mRNA analysis in 10.5-day embryos from each line; samples chosen were those closest to the mean for embryos of that age in each line. The positions of mouse ζ (ζ^M) and α (α^M) and human ζ (ζ^H) and α (α^H) are shown. The protected bands of the human ζ probe normally run as a doublet. (*Middle*) Similar analysis of adult individuals of each line. The extraneous band below the α^H band in α28 (and more weakly in other samples) is residual full-length α^M probe. (*Bottom*) Copy-number analysis of first-generation mice in each line. *Pst* I-digested DNA was hybridized with probes specific for mouse erythropoietin (1.0-kb band, a control for the amount of DNA loaded) and human α-globin (1.6-kb band). A shorter exposure of the human α-globin gene signal is shown above to allow better estimation of the high-copy-number samples. H101 is a murine erythroleukemia hybrid cell line with a single copy of human chromosome 16 containing two α genes ($\alpha\alpha$). Lanes labeled 1 to 64 represent dilutions of a plasmid containing the human α-globin gene in mouse DNA and calibrated to contain 1 to 64 copies.

When the expression of the human α (and ζ) gene was plotted versus gestational age (Fig. 3) three patterns emerged, unrelated to the constructs. In three lines (α30, $\alpha 37_H$, and α40) expression levels were similar in embryos and adults. Lines α28, α33, $\alpha 37_L$, α42, α43, α45, and α49 (as well as the previously reported lines α11, α12, and α13) showed an $\approx$3-fold drop in the level relative to mouse α between 10.5-day embryos and adults. The third pattern, seen in lines α22, α29, α32, and α44, was an even more extreme repression of human gene expression, with a >10-fold decrease in human globin mRNA between the embryonic and adult stage (Fig. 4).

When the total human globin mRNA levels in 10.5-day embryos were compared between the different constructs, the results for the HS $-40\zeta\alpha$ and 4HS$\zeta\alpha$ constructs were similar (44.0%) to those previously obtained with HS -40α (39.7%); slightly lower levels were obtained with cNFGα, with a mean of 21.6%.

Relationship of Copy Number to α-Globin mRNA Expression. The relationship between copy number and expression is complicated by the change in expression levels during development. In adults, expression per copy in the cNFGα and HS $-40\zeta\alpha$ lines tended to decrease with increasing copy number, as seen for HS -40α, whereas with the 4HS$\zeta\alpha$ construct, it remained relatively constant across a >10-fold range of copy number, indicating that expression increases with copy number in a fairly linear fashion (Table 1). This relationship was not observed in embryos, where the 4HS$\zeta\alpha$ lines showed the same inverse correlation between copy number and expression per copy as seen with the other constructs. Indeed, when human mRNA expression per copy was plotted for all lines, the data from the embryonic samples all fell on more or less the same curve (Fig. 5). It appears, therefore, that the additional HSs present in the 4HS$\zeta\alpha$ construct do not result in increased expression over the constructs containing HS -40 alone, at any copy number. Furthermore, these data confirm that at low copy numbers, the expression of the human transgene in embryos approaches that of the endogenous mouse genes.

Developmental Regulation of the ζ- and α-Globin Genes. Human ζ-globin gene expression was examined in the six lines containing the HS $-40\zeta\alpha$ or 4HS$\zeta\alpha$ construct. Human ζ mRNA levels were approximately equal to or exceeded human α mRNA at 8.5–9.5 days of gestation (Fig. 4). Thereafter, the proportion of ζ mRNA declined until it became undetectable at 14.5 days of gestation, closely following the pattern of mouse ζ gene expression (Fig. 6). The mean human α/mouse $\alpha + \zeta$ mRNA level in these six lines was 31.3% at 10.5 days of gestation, while the level in the 11 cNFGα and HS -40α lines, which lack the human ζ gene, was 26.5%. This suggests that the level of human α mRNA transcription

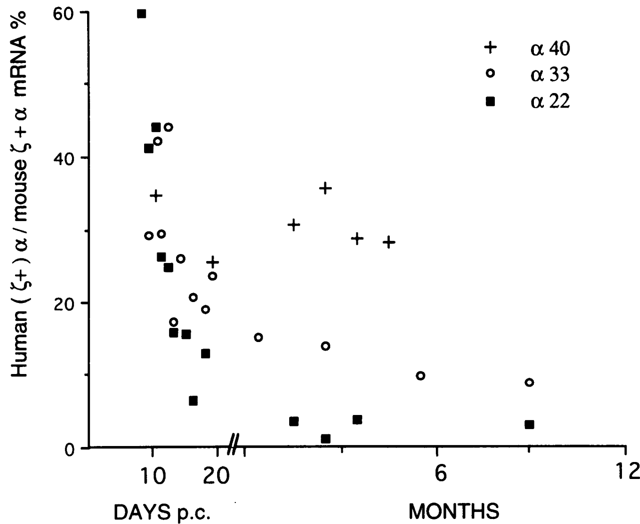

FIG. 3. Representative patterns of human globin gene expression during development in three transgenic lines. Each point during embryonic and fetal development represents the mean of 3–12 individual embryos; the range is omitted for clarity but in general was within 20% of the mean. Values given for postnatal samples represent the mean of up to 6 animals.

is not reduced by the presence of the ζ gene in constructs bearing both genes.

DISCUSSION

Previous studies have demonstrated that HS −40 is a major regulator of α-globin gene expression, but it is not clear whether or not this is the only regulatory element required for maximum gene activity (14–16). We have tested, therefore, additional upstream HSs for their effect on human α-globin gene expression in transgenic mice.

The results have confirmed that only fragments containing HS −40 are active in this system. The cRN24α construct, containing HS −4, HS −8, and HS −10, produced barely detectable levels of α mRNA in transgenic mice. In contrast, the 14 new lines bearing constructs containing HS −40 showed readily detectable levels of human α mRNA and enabled us to assess whether the additional HSs had any effects on (*i*) the developmental persistence of human gene expression, (*ii*) copy number-dependent expression, and (*iii*) the overall expression level.

There was clearly a tendency in most lines for expression levels to be higher in embryos than adults, up to 20-fold in some cases. The cause of this remains unknown. A similar pattern of decreasing human gene expression during development has been reported for β gene constructs containing HS2 (30) but not in mice bearing the whole LCR–β-globin cluster as a single 70-kb fragment (29).

Expression levels in the mice studied here were not copy-number dependent, even at the low end of the range, but in general showed an inverse relationship between copy number

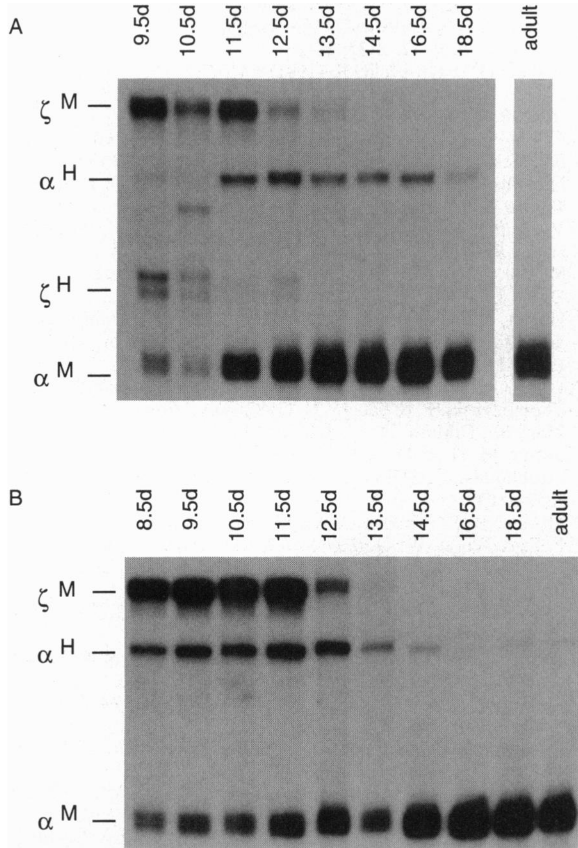

FIG. 4. Expression of mouse (M) and human (H) ζ- and α-globin mRNA during development in two transgenic lines: α22 (4HS ζα) (*A*) and α29 (cNFGα) (*B*).

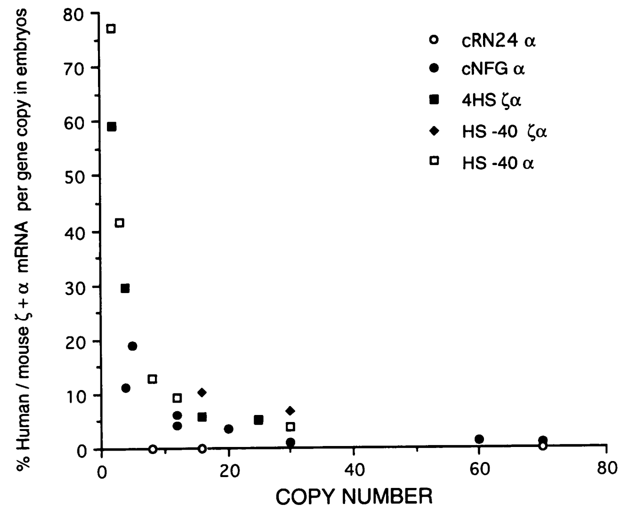

FIG. 5. Human α mRNA expression per gene copy, as a percentage of mouse gene expression, versus copy number for each of the transgenic lines.

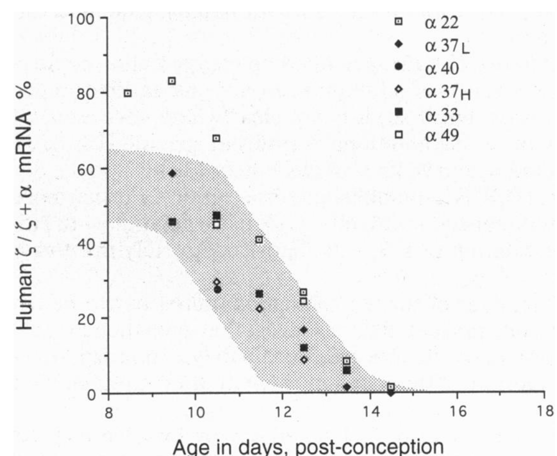

FIG. 6. Pattern of human ζ-globin gene expression in the six lines containing both genes. Shaded area represents the range of the mouse ζ/(ζ + α) mRNA ratio in the same lines.

and expression per copy. This was most clearly seen when the expression levels observed in embryos were used to calculate the expression per copy but also held for the HS -40α, HS $-40\zeta\alpha$, and cNFGα adult data.

Despite these complications there did not appear to be a significant increase in expression or in expression per copy between animals with the construct containing four HSs and those bearing only HS −40. No significant differences were seen in the mean expression levels in adults (21.4% vs. 18.1%), expression per copy in adults (4.8% vs. 6.0%), expression levels in embryos (39.7% vs. 38.7%), or expression per copy in embryos (18.1% vs. 14.5%). Furthermore, when expression per copy in embryos is plotted against copy number, the points for each construct appear to lie on the same line; in other words, at equivalent copy numbers, expression levels are very similar (Fig. 5).

Results from the HS $-40\zeta\alpha$ and 4HS$\zeta\alpha$ lines demonstrate that the human ζ gene is developmentally regulated in transgenic mice. Previously it has been shown that the human ζ gene under the control of β-LCR elements is expressed only in embryonic cells of transgenic mice (31, 32) and that expression of a ζ promoter–*lacZ*–HS −40 construct was developmentally regulated in such mice (33). Our results demonstrate that, both qualitatively and quantitatively, the expression of the human ζ gene relative to the α gene follows a pattern identical to that of the endogenous mouse genes. Thus at the earliest time points examined ζ mRNA predominated but some α mRNA was always observed. It does not appear that the α genes are silent at any stage of mouse development, an observation which has been confirmed by *in situ* hybridization (34).

The lack of copy-number dependence and the decline in α gene expression during development suggest that HS −40 is not equivalent to the β-LCR. While three, if not all four, HS elements are necessary for maximum expression of the β-like genes, we have no evidence that additional sequences in the region upstream of the α-globin genes contribute to expression levels.

The lack of complete regulation of the human α genes in transgenic mice could be due either to cross-species incompatibility or to the lack of additional regulatory sequences which have yet to be identified around the human α-globin gene cluster. Transcription involves the interaction of trans-acting factors with cis-active sequences in the enhancers and promoters of genes. These interactions are sequence specific, since single base substitutions can affect expression levels and developmental specificity of the globin genes (35–37). Since there are sequence differences in the α and ζ gene promoters between humans and mice, the interaction of mouse transcription factors with human sequences may be suboptimal.

Alternatively, the lack of complete regulation could be due to the absence of additional, as yet unidentified, regulatory sequences, but if so, it is not clear where such elements are likely to lie. No additional, erythroid-specific HSs have been detected within 90 kb 5′ of HS −40 or 120 kb 3′ to the α-globin genes (D.R.H., unpublished observation). Furthermore, any such sequences could only work in association with HS −40, since deletion of HS −40 almost completely inactivates the genes.

Whichever of these explanations turns out to be correct, the results presented here suggest that even though the α- and β-globin gene clusters are coordinately expressed, there may be important differences in the way they are regulated.

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