Proximal promoter elements of the human \( \zeta \)-globin gene confer embryonic-specific expression on a linked reporter gene in transgenic mice

M. D. Pondel 1,*, J. A. Sharpe 2, S. Clark, L. Pearson, W. G. Wood 2 and N. J. Proudfoot

The Sir William Dunn School of Pathology, Chemical Pathology Unit, University of Oxford, Oxford OX1 3RE, UK, 1St George's Hospital Medical School, University of London, Department of Histopathology, Cranmer Terrace, Tooting, London SW17 ORE, UK and 2MRC Molecular Haematology Unit, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

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ABSTRACT

We have investigated the transcriptional regulation of the human embryonic \( \zeta \)-globin gene promoter. First, we examined the effect that deletion of sequences 5′ to \( \zeta \)-globin’s CCAAT box have on \( \zeta \)-promoter activity in erythroid cell lines. Deletions of sequences between –116 and –556 (cap = 0) had little effect while further deletion to –84 reduced \( \zeta \)-promoter activity by only 2–3-fold in both transiently and stably transfected erythroid cells. Constructs containing 67, 84 and 556 bp of \( \zeta \)-globin 5′ flanking region linked to a β-galactosidase reporter gene (lacZ) and hypersensitive site –40 (HS –40) of the human \( \alpha \)-globin gene cluster were then employed for the generation of transgenic mice. LacZ expression from all constructs, including a 67 bp \( \zeta \)-globin promoter, was erythroid-specific and most active between 8.5 and 10.5 days post-fertilisation. By 16.5 days gestation, lacZ expression dropped 40–100-fold. These results suggest that embryonic-specific activation of the human \( \zeta \)-globin promoter is conferred by a 67 bp \( \zeta \)-promoter fragment containing only a CCAAT and TATA box.

INTRODUCTION

The human haemoglobin molecule is encoded by genes within the \( \alpha \)- and \( \beta \)-globin gene clusters. The \( \alpha \)-cluster consists of three functional genes arranged 5′-\( \alpha 2 \)-\( \alpha 2 \)-\( \alpha 1 \)-3′ at the tip of chromosome 16p while the \( \beta \)-globin gene cluster consists of five functional genes arranged 5′-\( \beta 4 \)-\( \gamma 2 \)-\( \gamma 2 \)-\( \delta 2 \)-\( \delta 2 \)-3′ on chromosome 11p. The expression of genes within each cluster is regulated in a tissue- and developmental stage-specific manner to produce embryonic (\( \zeta 2 \)_E, \( \alpha 2 \)_E, \( \zeta 2 \)_P), foetal (\( \delta 2 \)_P) and adult (\( \alpha 2 \)_D and \( \alpha 2 \)_D) globins. While the mechanism(s) responsible for the co-ordinated expression of these genes is hypothesised to occur at the transcriptional level (1), little is known about the specific sequences within these genes that may play a role in the regulation of the switching process.

High level expression of the \( \alpha \)- and \( \beta \)-like globin genes in stably transfected cell lines and transgenic mice is dependent upon sequences located far upstream of each respective gene cluster.

The \( \beta \) locus control region (\( \beta \)LCR), is located 5–20 kb upstream of the \( \beta \)-globin gene locus and consists of four erythroid-specific DNase I hypersensitive sites (2,3). The \( \beta \)LCR can confer high level expression to a linked \( \beta \)- or \( \alpha \)-globin gene in a position-independent, copy number-dependent manner in transgenic mice (2–6). A region with some of these properties has been localised 40 kb 5′ to the human \( \alpha \)-globin gene cluster. Like the \( \beta \)LCR, this region (HS –40) confers high levels of erythroid-specific expression to a linked \( \alpha \)- or \( \zeta \)-globin gene in transgenic mice (7–10).

When a 70 kb fragment containing the whole of the \( \alpha \)-globin cluster including HS –40 is expressed in transgenic mice, the human \( \zeta \)-globin gene is developmentally regulated and matches that of the endogenous mouse \( \zeta \)-globin gene (11). This correct developmental regulation is also seen when the \( \zeta \)-globin gene is attached to an HS –40\( \alpha \) gene fragment (9) as well as with \( \mu \)LCR and \( \beta \)LCR HS2 constructs (12,13). These results suggest that developmental regulation is controlled by sequences in and around the \( \zeta \)-globin gene itself.

Deletions of the \( \zeta \)-globin gene promoter in HS2\( \zeta \) constructs have suggested that as little as 128 bp of the promoter are sufficient to confer embryonic expression on this gene (14). Furthermore, we and others have demonstrated that a 556 bp \( \zeta \)-globin promoter fragment is sufficient to confer embryonic-specific expression to a linked lacZ reporter gene (10,15). The sensitivity of this assay, together with its ability to provide quantitative data on expression levels and intercellular variability, led us to use it to better define the promoter sequences that confer embryonic expression.

In the present study, we first examined the effect that deletion of progressive amounts of \( \zeta \)-globin 5′ flanking region had on \( \zeta \)-globin gene expression in transiently transfected versus stably integrated erythroid cells. \( \zeta \)-globin promoter/lacZ deletion constructs were then employed for the generation of transgenic mice followed by analysis of lacZ staining pattern and expression levels in transgenic embryos and foetuses.

MATERIALS AND METHODS

DNA constructs

To produce \( \zeta \)-globin/CAT constructs containing varying amounts of \( \zeta \)-globin 5′ flanking region, Bal31 digestion of \( \zeta \)-globin’s 5′ flanking region was employed as previously described (16).
Hin and late promoter driving Neo resistance was cloned into the erythroid cell lines, a 2.7 kb fragment containing the SV40 early construct. To employ these constructs for the generation of stable Pst(10) (Fig. 3 B). To produce the construct then excised with StyI restriction fragment containing 86 bp of \(\zeta\)-globin 5′ flanking region (cap = 0) was ligated into the \(\zeta\)-globin promoter/CA T/HS –40 constructs containing (B) 0, (C) 556, (D) 195, (E) 116 and (F) 84 bp of \(\zeta\)-globin 5′ flanking region.

\(\zeta\)-globin 5′ flanking regions containing 84, 116, 195 or 556 bp of DNA (cap = 0) were ligated into the SmaI site of the vector pCATO (16). A 4 kb HindIII fragment (BamHI linker) containing HS –40 was then cloned into the BamHI site of each construct. To employ these constructs for the generation of stable erythroid cell lines, a 2.7 kb fragment containing the SV40 early and late promoter driving Neo resistance was cloned into the HindIII sites of the above CAT constructs (Fig. 1B).

The construct \(\zeta\)556/lacZ/HS –40 has previously been described (10) (Fig. 3B). To produce the construct \(\zeta\)84/lacZ/HS –40, a PstI–StyI restriction fragment containing 86 bp of \(\zeta\)-globin 5′ flanking region (cap = 0) was isolated from the vector pCATEZ (16). The fragment was blunt-ended with T4 DNA polymerase and cloned into the SmaI site of pGEM 7Z(f)+. The insert was then excised with StyI–EcoRI, blunt-ended with Klenow and cloned into the blunt-ended HindIII site of the vector lacZ/HS –40 (Fig. 3C).

To produce the construct \(\zeta\)67/lacZ/HS –40, complementary oligonucleotides containing an Asp718 site followed by 67 bp of \(\zeta\)-globin 5′ flanking region (cap = 0) were annealed and ligated into the blunt-ended HindIII site of the vector lacZ/HS –40 (Fig. 3D).

The construct \(\alpha\)575/lacZ/HS –40 was produced by first excising a 575 bp human \(\alpha\)-globin promoter fragment (cap = 0) cloned into the HindII/PstI site of pUC9 with PvuII–BamHI. The fragment was blunt-ended with T4 DNA polymerase and cloned into the blunt-ended HindIII site of lacZ/HS –40 (Fig. 3E).

**Figure 1.** (A) Human \(\alpha\)-globin gene locus showing the position of erythroid specific HS –40 (E.H.S.) and the sequence of 88 bp of human \(\zeta\)-globin 5′ flanking region. \(\zeta\)-globin promoter/CAT/HS –40 constructs containing (B) 0, (C) 556, (D) 195, (E) 116 and (F) 84 bp of \(\zeta\)-globin 5′ flanking region.

**Generation and screening of transgenic mice**

Transgenic mice were generated by micro injection of linear DNA fragments into pronuclei of fertilised eggs from CBA \(\times\) C57 crosses (17). Transgenic progeny were identified and copy number of transgene determined as previously described (10). Hemizygous lines were established by mating transgenic founders to CBA \(\times\) C57 F1 mice.

To generate transgenic foetuses containing an \(\alpha\)-globin promoter/lacZ construct, \(\alpha\)575/lacZ/HS –40 was cut with Asp718 and HindIII. The released fragment was purified as previously described (10) and injected into fertilised F1 eggs followed by embryo transfer into pseudo-pregnant pathology outbred (PO) mice. After 16.5 days of gestation, the foetuses were removed and subjected to lacZ analysis (see below).

**Histochemical analysis of \(\zeta\)-promoter activity in transgenic embryos and foetuses**

Transgenic males from each line were mated to female wild-type F1 mice. The appearance of a vaginal copulation plug was considered day 0.5. At day 8.5–9.5 post-fertilisation, whole embryos were fixed and assayed for lacZ activity employing 5-bromo-4-chloro-3-indoyl-\(\beta\)-D-galactopyranoside (X-gal) as previously described (10). To assay for \(\zeta\)-globin promoter activity at day 16.5, transgenic foetuses were identified, their livers removed, fixed and subjected to lacZ analysis as above. Whole embryos and livers were photographed on a dissection microscope. For more detailed histochemical analysis, embryos and livers were embedded in paraffin, sectioned (5 \(\mu\)m) and counter stained with cresyl violet or eosin.

To analyse \(\alpha\)575/lacZ/HS –40 expression, livers from 16.5 day old transgenic foetuses were removed and fixed, followed by incubation in X-gal as previously described (10).

To determine the percentage of lacZ positive cells in the blood of transgenic embryos and foetuses containing \(\alpha\)– or \(\zeta\)-globin/ lacZ/HS –40 constructs, peripheral blood from 10.5 and 16.5 day old embryos and foetuses was isolated and stained with X-gal as previously described (15). The number of lacZ positive cells were counted employing a haematocytometer.

**Quantitation of \(\zeta\)-promoter/lacZ/HS –40 expression during development**

In order to quantify \(\zeta\)-promoter/lacZ/HS –40 expression levels during development, peripheral blood from 10.5–16.5 day old transgenic embryos and foetuses was obtained as above. Blood cells were pelleted by centrifugation and re-suspended in 250 mM Tris pH 7.5. The cells were subjected to freeze–thaw three times followed by a 10 min centrifugation in a microfuge. The supernatant was removed and assayed for protein concentration using a BioRad protein assay kit. Analysis of lacZ activity in extracts was performed as previously reported (10).

To quantitate lacZ activity in \(\alpha\)575/lacZ/HS –40 transgenic foetuses, blood from 16.5 day old transgenic foetuses was isolated and lacZ activity assayed as above.

**Tissue culture and transfection**

K562 cells were maintained in DMEM supplemented with 10% foetal calf serum, 100 \(\mu\)g/ml penicillin, 100 U/ml streptomycin and 2 mM glutamine. Putko cells were maintained in RPMI 1640 supplemented with 10% foetal calf serum, 100 \(\mu\)g/ml penicillin and 100 U/ml streptomycin.

Pools of K562 clones stably transfected with \(\zeta\)-promoter/lacZ/HS –40 Neo constructs were generated as previously described (18). For transient transfections, Putko cells were electroporated with \(\zeta\)-promoter/lacZ/HS –40 constructs as previously described (19). Plasmid pIRV (20) (5 \(\mu\)g) was employed as a co-transfection.
control. Forty-eight hours after transfection, cells were harvested and extracts produced as above. CAT and lacZ analysis was performed as outlined in Pondel et al. (19).

RESULTS

ζ-globin promoter activity in transiently and stably transfected erythroid cell lines

We previously showed that deletion of sequences 5′ to the human α-globin CCAAT box caused a significant decrease in α-globin promoter activity when linked to HS −40. Interestingly, this decrease in expression occurred in stably but not transiently transfected cells (18). In order to determine how much sequence 5′ to the ζ-globin gene was required to give readily detectable expression, ζ-globin/CAT/HS −40 deletion constructs were employed for the generation of stably transfected erythroid cell lines (Fig. 1B–F). Since HS −40 does not confer complete position independent expression to a linked ζ-globin gene (8–10, 15), CAT assays were performed on extracts from pools of G418 resistant clones (>100 clones per pool). By analysing ζ-globin activity from complex pools of clones, the effect position of integration has on ζ-promoter activity should be averaged out amongst the clones. The results of these experiments are depicted in Figure 2. Deletion of sequences between −116 and −556 (cap = 0) caused no significant decrease in ζ-promoter/CAT/HS −40 activity. When an additional 32 bp were deleted (ζ84/lacZ/HS −40), a 2–3-fold drop in promoter activity was observed.

When the above constructs (minus SV40-Neo) were transiently transfected into erythroid cells, the effect of the deletions on ζ-globin promoter activity was similar to that found in stably transfected cells. This contrasts results reported by Sabath et al. (21) who found that the deletion of sequences between −207 and −417 caused a 95% decrease in ζ-promoter activity in transiently transfected erythroid cells. These conflicting results may be due to the use of different reporter genes or different cell lines. As our results suggest that sequences 5′ to ζ-globin’s CCAAT box may not be required for high level expression of a reporter gene in either transiently or stably transfected erythroid cells, we examined the expression of similar small promoter constructs in transgenic mice.

Histochemical analysis of lacZ expression in transgenic embryos and foetuses

In the developing mouse, erythropoiesis first occurs in yolk sac blood islands between 8 and 14 days gestation. Mouse ζ-globin expression at this site reaches its peak at ~9.5 days of development and then gradually decreases to almost undetectable levels by 15–16 days of gestation (22). We previously showed that 556 bp of human ζ-globin 5′ flanking region linked to lacZ and HS −40 was sufficient to direct a similar pattern of lacZ activity in transgenic mice (10). In order to more clearly define sequences that direct embryonic specific expression of the human ζ-globin gene, transgenic lines containing 556, 84 or 67 bp of human ζ-globin 5′ flanking region linked to lacZ and HS −40 were generated (Fig. 3). Southern blot analysis identified six transgenic lines containing 12–150 copies of the ζ84/lacZ/HS −40 construct and nine transgenic lines containing 67/lacZ/HS −40 construct (Table 1A). Embryos from three lines containing ζ84/lacZ/HS −40 and two lines containing the ζ67/lacZ/HS −40 construct showed no lacZ activity (Table 1A). Southern blot analysis did not reveal any obvious rearrangement or deletion of the above constructs in these mice suggesting that the absence of ζ-promoter activity is due to position effects. Two transgenic lines containing 12 copies of ζ556/lacZ/HS −40 (10) were employed as a control for correct developmental regulation.

The results of X-gal staining of transgenic embryos can be seen in Figure 4. In 8.5 day old transgenic embryos containing ζ67/lacZ/HS −40 or ζ556/lacZ/HS −40, lacZ expression formed a ring around the yolk sac corresponding with the position of blood islands formed at this stage of development (Fig. 4A and D). By 9.5 days post-fertilisation, lacZ activity was observed in the blood vessels covering the yolk sac of embryos with either construct (Fig. 4B and E) as well as in the blood vessels of each
Figure 4. Histochemical analysis of lacZ expression during embryonic development. X-gal staining of whole embryos containing ζ556/lacZ/HS–40 at (A) 8.5 days and (B and C) 9.5 days. X-gal staining of embryos containing ζ67/lacZ/HS–40 at (D) 8.5 days and (E and F) 9.5 days. (G and H) Sections (5 μm) from 9.5 day old embryos containing ζ556/lacZ/HS–40 or ζ67/lacZ/HS–40, respectively. MD, maternal decidua; YS, yolk sac; H, heart.
embryo (Fig. 4C and F). After sectioning, staining was again observed to be limited to the erythroid cells in the yolk sac, the heart and various blood vessels (Fig. 4G and H). Transgenic embryos that contained the ζ84/lacZ/HS –40 construct showed an identical pattern of expression (data not shown).

The suppression of mouse ζ-globin expression during development is associated with a shift in the major site of erythropoiesis from the yolk sac to the foetal liver. We previously observed that expression from ζ575/lacZ/HS –40 was suppressed in the foetal liver of transgenics (10). To determine if expression from ζ67/lacZ/HS –40 and ζ84/lacZ/HS –40 was also suppressed at foetal stages of development, livers from 16.5 day of gestation in pseudo-pregnant PO females were removed. Expression dropped on average, 11-fold. An additional 7-fold drop in lacZ activity occurred between 14.5 and 16.5 days of development. LacZ expression in peripheral blood of all transgenic lines dropped significantly. In contrast, peripheral blood from transgenic foetuses containing α575/lacZ/HS –40 showed abundant levels of lacZ expression in cell lysates (Table 1B).

To carry out a more detailed analysis of ζ promoter activity during development, additional lacZ analysis was performed on five high-level lacZ expressing transgenic lines at four developmental time points (Fig. 6). Peripheral blood lysates from all transgenic lines analysed showed a 2–3-fold reduction in lacZ reporter was observed in embryonic erythroblasts in several lines (Table 1A). Expression levels in each line did not appear to be correlated with copy number. However, statistical analysis revealed a significant correlation (r² = 0.911) between the number of lacZ positive cells and lacZ expression levels in 10.5 day old transgenic embryos. By 16.5 days of development, lacZ expression in peripheral blood of all transgenic lines dropped significantly. In contrast, peripheral blood from transgenic foetuses containing α575/lacZ/HS –40 showed abundant levels of lacZ expression in cell lysates (Table 1B).

Quantitative analysis of lacZ expression in transgenic embryos and foetuses

To determine the degree to which ζ promoter activity was suppressed at foetal stages of development, peripheral blood from transgenic embryos and foetuses was isolated. Half of each sample was employed for histochemical staining and the other half for quantitative β-galactosidase assays. We observed a marked variation (0.1–100%) in the percentage of lacZ expressing erythroid cells in 10.5 day old embryos from different transgenic lines (Table 1A). This variation was consistent even after overnight incubation of blood in X-gal. By day 16.5 post-fertilisation, the percentage of lacZ positive cells in peripheral blood from all transgenic lines dropped significantly. In contrast, day 16.5 peripheral blood from all transgenic foetuses containing the α575/lacZ/HS –40 construct showed high proportions of lacZ positive cells (Table 1B).

Robertson et al. (15) reported that expression of ζ-promoter/lacZ/HS –40 constructs in erythroid cells of transgenic mice was bi-modal (on/off). In contrast, we see heterogeneity of intercellular lacZ expression (data not shown) in all of our transgenic mice. This suggests that ζ-promoter activity is variable in the erythroid cells of any given transgenic mouse.

LacZ assays on peripheral blood lysates showed that ζ-globin promoter activity was highly variable between different transgenic lines (Table 1A). Expression levels in each line did not appear to be correlated with copy number. However, statistical analysis revealed a significant correlation (r² = 0.911) between the number of lacZ positive cells and lacZ expression levels in 10.5 day old transgenic embryos. By 16.5 days of development, lacZ expression in peripheral blood of all transgenic lines dropped significantly. In contrast, peripheral blood from transgenic foetuses containing α575/lacZ/HS –40 showed abundant levels of lacZ expression in cell lysates (Table 1B).

DISCUSSION

During normal mouse development, expression of the ζ-globin gene is essentially limited to the primitive erythroid cells produced in the blood islands of the embryonic yolk-sac. The results presented here demonstrate that as little as 67 bp of the human ζ-globin promoter, in the presence of HS –40, appears to be sufficient to confer embryonic stage specificity on a linked lacZ reporter gene. Erythroid specific, high level expression of the reporter was observed in embryonic erythroblasts in several lines of transgenic mice. LacZ expression levels declined 40–100-fold by day 16.5 of gestation. These results extend those of Sabath et al. (14) who obtained similar results with a ζ-globin gene containing 128 bp of ζ-globin 5′ flanking region, albeit under the control of the βLCR HS2.

Robertson et al. (23) have also used ζ-promoter/lacZ/HS –40 constructs and reported a much smaller decrease in expression (only 1.5–15-fold) between days 12.5 and 17.5 with the ζ-globin promoter truncated to either 556 or 84 bp. However, in these cases, the HS –40 fragment was in the opposite orientation.
Figure 5. Histochemical analysis of lacZ expression in foetal livers. X-Gal staining in whole livers and 5 µm sections from transgenic foetuses containing (A and B) ζ67/lacZ/HS –40 (C and D) ζ556/lacZ/HS –40 (E and F) α575/lacZ/HS –40.
relative to the $\zeta$-promoter/lacZ fragment. When the HS –40 fragment was in the same 5’–3’ orientation as the reporter gene (550CR in their nomenclature), the decline in lacZ expression in their mice was similar to that in ours. As HS –40 has been shown to be orientation independent (7), it seems unlikely that this result would bring the core enhancer sequences much closer (1.0 versus ~3.5 kb) to the $\zeta$-globin gene promoter of the next copy downstream in a tandem head-to-tail array. We would suggest, therefore, that perhaps the close proximity of the enhancer to the promoter has partially overridden the developmental control mechanism in the studies of Robertson et al. (23).

Liebhaber et al. (24) have recently suggested that sequences in the $\zeta$-globin promoter, the transcribed portion of the gene and 3’ to the gene are necessary for complete silencing of the human $\zeta$-globin gene in post-embryonic transgenic mice. When the 557 bp $\zeta$-globin promoter was attached to an $\alpha$-globin gene there was only a 2.7-fold drop in expression between 9.5 and 16.5 days as opposed to a 50-fold drop with the intact $\zeta$-globin gene. However, this result does not preclude the possibility that the $\alpha$-globin gene contributes sequences that oppose the silencing effects of the $\zeta$-promoter. Furthermore, these studies used the $\beta$-globin mLCR closely apposed to the $\zeta$-globin promoter and again this could affect the normal pattern of developmental regulation.

Watt et al. (16) showed that in the absence of HS –40, GATA-1 binding sites present in the 5’ flanking region of the $\zeta$-globin promoter direct its erythroid specificity. Our data shows that GATA-1 binding sites in the 5’ flanking region of the $\zeta$-globin promoter are not required for erythroid specific activity of the $\zeta$-globin promoter when it is linked to HS –40. Since HS –40 enhancer capability is erythroid-specific (7,19,25), we hypothesise that erythroid-specific expression of $\zeta\beta$/lacZ/HS –40 is mediated primarily by the HS –40 element.

A number of transcription factors bind to sequences within 67 bp of $\zeta$-globin 5’ flanking region. The factors CP1 or CP2 bind to the $\zeta$-globin CCAAT box (16,26). The $\zeta$-promoter also contains a TATA box, suggesting this region binds TATA binding protein and TATA box associated factors (TBP and TAF). Although the human $\alpha$-globin promoter also binds these proteins, it is transcriptionally active at all stages of development. Clearly, there are as of yet, unidentified sequences within the $\zeta$-globin proximal promoter that direct its embryonic-specific transcriptional activity. Alternatively, the $\zeta$-globin CCAAT and/or TATA box may be interacting with developmental stage specific transcription factors that regulate the switching process. Our delineation of a small region capable of directing correct temporal transcriptional activity of the $\zeta$-globin promoter should facilitate the identification of such sequences or core promoter elements. We will then be in a position to study their interaction with nuclear regulatory factors that play an important role in directing the $\zeta$- to $\alpha$-globin switch.

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