A 3'-flanking NF- κ B site mediates developmental silencing of the human ζ -globin gene

Zhibin Wang and Stephen A. Liebhaber¹

Howard Hughes Medical Institute and Departments of Genetics and Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

¹Corresponding author
e-mail: liebhaber@mail.med.upenn.edu

The central developmental event in the human (h) α -globin gene cluster is selective silencing of the ζ -globin gene as erythropoiesis shifts from primitive erythroblasts in the embryonic yolk sac to definitive erythroblasts in the fetal liver. Previous studies have demonstrated that full developmental silencing of the h ζ -globin gene in transgenic mice requires the proximal 2.1 kb of its 3'-flanking region. In the current report, we localize this silencing activity to a 108 bp segment located 1.2 kb 3' to the ζ -globin gene. Protein(s) in nuclear extracts from cell lines representing the fetal/adult erythroid stage bind specifically to an NF- κ B motif located at this site. In contrast, this binding activity is lacking in the nuclear extract of an embryonic-stage erythroid line expressing ζ -globin. This complex is quantitatively recognized by antisera to the NF- κ B p50 and to a lesser extent to p65 subunits. A two-base substitution that disrupts NF- κ B site protein binding *in vitro* also results in the loss of the developmental silencing activity *in vivo*. The data suggest that NF- κ B complex formation is a crucial component of h ζ -globin gene silencing. This finding expands the roles of this widely distributed transcriptional complex to include negative regulation in mammalian development.

Keywords: embryonic expression/ ζ -globin gene/gene silencing/NF- κ B/transcription factor

Introduction

The human (h) α -globin gene cluster, located on chromosome 16p, contains three expressed genes arranged in the order of their developmental expression; 5' ζ - α 2- α 1 3' (Figure 1A; also see Higgs *et al.*, 1989). h ζ -globin is selectively expressed in the primitive erythroid cells of the embryonic yolk sac where it assembles into the embryonic hemoglobin tetramers, $\zeta_2\epsilon_2$ and $\zeta_2\gamma_2$. Concurrent with the shift to definitive erythropoiesis in the fetal liver, occurring at gestational weeks 6–7 in humans and days 9–12 in mice, expression of the ζ -globin gene is suppressed to trace levels (Peschle *et al.*, 1985; Albitar *et al.*, 1989). In contrast, the two adjacent α -globin genes, α 1 and α 2, are co-expressed throughout development; they are initially activated in the primitive erythroid cells and their expression is subsequently increased several fold as the ζ -globin genes are silenced (Albitar *et al.*, 1992).

The mechanism(s) responsible for switching within the α -globin gene cluster, and specifically for selective silencing of the h ζ -globin gene at the embryonic-to-fetal transition of erythropoiesis, remains undefined.

Transgenic mice constitute an informative model system for the study of switching in the h α -globin gene cluster. The murine (m) α - and h α -globin gene clusters are conserved in their structures and developmental profiles; both clusters contain a single embryonic ζ - and two co-expressed α -globin genes (Pressley *et al.*, 1980; Liebhaber *et al.*, 1981). Therefore human α - and ζ -globin transgenes can be directly compared with the corresponding set of endogenous mouse genes during development. Previous studies have revealed normal developmental control of the human α -globin gene cluster in the transgenic mice (Gourdon *et al.*, 1994). The developmental control of the h ζ -globin expression is gene-autonomous; all the necessary information for appropriate developmental silencing is present within and/or directly flanking the ζ -globin transgene and this control does not require *cis* interactions with the fetal/adult α -globin gene (Albitar *et al.*, 1991; Sabath *et al.*, 1993). Linkage of the ζ -globin transgene to either the major α -globin cluster regulatory element (HS-40) or the β -globin cluster locus control region (micro β -LCR or HSII) establishes a permissive chromatin domain and overcomes site-of-integration effects on the transgene (Albitar *et al.*, 1991; Sabath *et al.*, 1993; Sharpe *et al.*, 1993). A normal developmental profile of the h ζ -globin transgene is maintained irrespective of which of these two auxiliary elements is used to enhance transgene expression (Albitar *et al.*, 1991; Starck *et al.*, 1994; Hug *et al.*, 1996; Martin *et al.*, 1996). The parallel organization of the murine and human α -globin gene clusters and the gene-autonomous nature of h ζ -globin developmental control suggest that transgenic studies aimed at defining the *cis* determinants of h ζ -globin gene silencing may be highly informative.

The transcriptional activities of many structural genes are controlled by DNA sequences within the promoter and/or in the contiguous 5'-flanking region. Consistent with this generality, the h ζ -globin gene with 550 bp of the proximal promoter was found to be appropriately silenced in transgenic mice (Pondel *et al.*, 1992; Sabath *et al.*, 1993, 1995). Subsequent 5'-deletional mapping of this region revealed that a h ζ -globin transgene with as little as 128 bp of 5'-flanking sequence is also normally regulated (Sabath *et al.*, 1993). However, extensive subsequent analysis of this promoter region failed to reveal elements that mediate silencing independently of global effects on transcription (Sabath *et al.*, 1993). These studies suggest that *cis*-acting determinants required for stage-specific expression of the h ζ -globin gene may reside within the transcribed or 3'-flanking sequences.

Recent studies suggest that silencing of the h ζ -globin

Fig. 1. Developmental profiles of the $h\zeta$ -globin transgenes linked to HS-40. (A) The human α -globin gene cluster. The three functional genes (ζ , $\alpha 2$ and $\alpha 1$) are shown as filled boxes, the three pseudogenes as open boxes and the θ gene, the expression status of which is unclear, as an open box. The developmental specificity of each of the expressed genes is noted above the cluster. The position of the α -globin locus major regulatory element, HS-40, located 40 kb upstream from the ζ -globin gene, is noted. (B) Structures of the HS-40 ζ Eco and HS-40 ζ transgenes. The full-length ζ -globin gene on a 4.4 kb *Eco*RI fragment contains three exons (filled boxes). HS-40 is located on a 1.4 kb *Eco*RI–*Hind*III fragment. Nomenclature denotes the extent of 3'-flanking region. The angled arrow marks the site of transcription initiation. (C) RNase protection assay of embryonic and fetal erythroid RNA samples from representative transgenic mouse lines. The gestational age of each embryo and fetus is noted; day 9.5 samples are from individual yolk sacs, and day 14.5 and day 16.5 samples are from individual fetal livers. The origins of samples from transgenic (+) or non-transgenic (–) litter mates are indicated. The autoradiograph panels show analyses with probes corresponding to α -globin mRNA ($m\alpha$), ζ -globin mRNA ($m\zeta$) and ζ -globin mRNA ($h\zeta$). The 150 nucleotide protected fragment corresponds to expression of the ζ -globin transgene; the 179 and 193 nucleotide protected fragments correspond to expression of the endogenous $m\alpha$ - and $m\zeta$ -globin mRNAs, respectively (Liebhaber *et al.*, 1996). (D) Expression of HS-40 ζ and HS-40 ζ Eco transgenes. The levels of transgene expression from five HS-40 ζ lines and two HS-40 ζ Eco lines are shown. Each value represents the average of at least two determinations carried out on tissues from three or more mice of each line at each time-point. Expression levels are calculated as a percentage of the combined expression of endogenous $m\zeta$ - and $m\alpha$ -globin genes corrected for specific activities of the respective probes (see Materials and methods). The developmental age of each sample is as days p.c. The transgene copy number for each line is shown in the box to the right of the respective name. The vertical arrow in each frame indicates the mean fold-decrease for each transgenic construct between day 9.5 and day 16.5.

gene is a complex process reflecting both transcriptional and post-transcriptional mechanisms. Using transgenic models, silencing determinants have been identified in both the transcribed region and the 3'-flanking region of the $h\zeta$ -globin gene (Liebhaber *et al.*, 1996). Replacement of the coding region of the $h\alpha$ -globin transgene with that of the $h\zeta$ -globin gene results in partial gene silencing despite the presence of the intact α -globin promoter. This silencing activity of the ζ -globin transcribed region has been linked to the inability of its 3'UTR sequences to stabilize ζ -globin mRNA in the prolonged post-transcription stage of fetal/adult erythroid maturation (Russell *et al.*, 1997, 1998). A second determinant of ζ -globin gene silencing has been localized to its 3'-flanking region. Previous studies using transgenic mouse models demonstrate that the $h\zeta$ -globin transgene containing 2.1 kb of contiguous 3'-flanking sequences exhibits proper

developmental regulation with a 50-fold decrease in ζ -globin mRNA levels from day 9.5 yolk sac to day 16.5 fetal liver (Liebhaber *et al.*, 1996). In contrast, truncation of the 3'-flanking region to 106 bp 3' to the poly(A) addition site significantly blunted silencing to a 10-fold decrease over the same developmental window (Liebhaber *et al.*, 1996). The transcriptional silencing mediated by the 3' flanking region can be detected as early as day 8.5–9.5, suggesting that the ζ -globin gene undergoes active silencing soon after it is activated. Thus, the combined effects of transcriptional silencing by the 3'-flanking region and accelerated clearance of residually expressed ζ -globin mRNA can account for most if not all of the developmental control observed for the intact ζ -globin transgene (Liebhaber *et al.*, 1996). While the *cis* and *trans* determinants of ζ -globin mRNA instability have been studied in detail (Russell and Liebhaber 1998), the basis

for silencing activity of the 3'-flanking region remains undefined. In the present report, we map, characterize and functionally test a *cis* determinant positioned within the 3'-flanking region of the h ζ -globin gene, which mediates developmental silencing.

Results

A developmental silencing determinant is located 3' to the human ζ -globin gene

We have previously demonstrated that the h ζ -globin transgene containing 0.55 kb of 5'-flanking sequences and 2.1 kb of 3'-flanking sequences is silenced 50-fold during the transition from primitive erythropoiesis (day 9.5 yolk sac) to definitive erythropoiesis (day 16.5 fetal liver) (Liebhaber *et al.*, 1996). This 50-fold decrease in the steady-state concentration of the transgene mRNA defines 'full developmental silencing' (see Materials and methods; Liebhaber *et al.*, 1996). This developmental control is markedly blunted by truncation of the 3' flanking region suggesting that an element(s) critical to full silencing is located in this region (see Introduction). The h ζ -globin transgenes in these prior studies were linked to the β -LCR to overcome site-of-integration effects (Talbot *et al.*, 1989). Prior to further mapping of the putative silencing determinant, we confirmed its function in the context of the homologous α HS-40 major regulatory element (Figure 1A). This was accomplished by comparing the expression profiles of the HS-40 ζ *Eco* transgene containing the entire 2.1 kb of contiguous 3'-flanking sequence, with the HS-40 ζ transgene that is truncated 106 bp 3' to the poly(A) addition site (Figure 1B). Multiple lines were established with each transgene and timed embryos were generated by crossing positive males (F₁ or greater) from each line with wild-type females. Total RNA was isolated from day 9.5 yolk sacs and day 16.5 fetal livers of the transgenic embryos, and globin mRNAs were quantified by an RNase protection assay (Liebhaber *et al.*, 1996). Representative studies are shown in Figure 1C. As expected, the α - and m ζ -globin genes were co-expressed in day 9.5 yolk sac while in day 16.5 fetal liver the level of α -globin mRNA increased and m ζ -globin gene was silenced. Analysis of transgene mRNA revealed equivalent expression of the HS-40 ζ *Eco* and HS-40 ζ transgenes at day 9.5, while at day 16.5 the HS-40 ζ *Eco* transgene was fully silenced and the HS-40 ζ transgene continued to be expressed. Quantitative analysis of mRNA expression profiles in five HS-40 ζ and two HS-40 ζ *Eco* mouse lines confirmed this pattern. The two HS-40 ζ *Eco* lines were silenced 60- and 118-fold, respectively (Figure 1D, right), while developmental silencing of the HS-40 ζ transgenes was markedly blunted (mean 15-fold decrease) (Figure 1D, left). These data substantiated the previous assignment of a silencing determinant to the 3'-flanking region and confirmed equivalent developmental control of the h ζ -globin transgene in the presence of the major regulatory elements derived from either the α - or the β -globin gene clusters.

The silencing element maps to a 108 bp sequence located 1.2 kb 3' to the h ζ -globin gene

Full developmental silencing of the h ζ -globin gene requires one or more elements within the proximal 2.1 kb

of the 3'-flanking region (as described above). To facilitate subsequent studies, the sequence of this 3'-flanking region was determined (DDBJ/EMBL/GenBank accession No. AF078904). This sequence revealed the presence of two complete Alu elements in a head-to-head transcriptional orientation separated by 400 bp (Figure 2A). Native restriction sites were utilized to generate a set of 3'-truncation constructs: ζ *Nco*, ζ *Bst* and ζ *Dra*. These genes extend 0.93, 1.33 and 1.80 kb into the 3'-flanking sequences, respectively (Figure 2A). Each gene was linked to the micro β -LCR (see above), because it resulted in a more reproducible and robust enhancement of overall globin transgene expression levels than the HS-40 (10-fold higher transgene transcript levels; see below). Transgenic founders were generated from each construct and Southern analysis of genomic DNA from respective F₁ progeny revealed the copy number and integrity of the transgenes. The developmental profile of the transgene in each line was determined using at least three individual embryos for each time-point. A study of a representative ζ *Nco* line demonstrated that transgene expression was present in day 9.5 yolk sac and persisted in day 16.5 fetal liver (Figure 2B, left). Quantitative analysis of all six ζ *Nco* lines revealed that silencing of the transgene expression was blunted in five of six lines (6- to 14-fold decrease with a mean drop of 11-fold; Figure 2C, left). This incomplete silencing paralleled that of the ζ -globin transgene with the fully truncated 3'-flanking region (Figure 1; Liebhaber *et al.*, 1996). These data suggested that the 3'-flanking region silencing element(s) was located more than 0.93 kb 3' to the h ζ -globin gene.

The expression profiles of the ζ *Bst* and ζ *Dra* transgenes were analyzed next. RNase protection assay of a representative ζ *Bst* line (Figure 2B, middle) revealed full silencing of mRNA expression, paralleling the endogenous m ζ -globin mRNA. Quantitative analysis of all four ζ *Bst* lines showed a remarkably consistent drop in transgene mRNA levels from day 9.5 yolk sac to day 16.5 fetal liver with a mean decrease of 45-fold (Figure 2C, middle). A similar result was found for the ζ *Dra* transgene with a more extensive 3'-flanking region; the mean silencing of transgene expression was 77-fold (Figure 2B right and C right). The expression profiles of the ζ *Nco*, ζ *Bst* and ζ *Dra* transgenes confirmed the presence of a silencing element in the 3' flanking region and mapped it within a 408 bp sequence bracketed by the *Nco*I and *Bst*1107I sites 3' to the h ζ -globin gene. Inclusion of this region was necessary for full (~50-fold) developmental silencing.

A second set of 3'-deletion transgenes was constructed to more precisely map the silencing element within the 408 bp *Nco*-*Bst* fragment. These transgenes contained 1.1, 1.2 and 1.3 kb of contiguous 3'-flanking sequences, respectively (ζ 1.1, ζ 1.2 and ζ 1.3; Figure 3A). RNA analysis of day 9.5 yolk sacs and day 16.5 fetal livers indicated incomplete developmental silencing of the ζ 1.1 transgene with a mean 10-fold decrease from day 9.5 yolk sacs to day 16.5 fetal livers (Figure 3B and C). In marked contrast, the ζ 1.2 transgene, containing an additional 108 bp, was fully silenced as was the ζ 1.3 transgene (mean decreases of 90-fold in both cases; Figure 3B and C). These results sublocalized the 3'-silencing element to an 108 bp fragment beginning 1.1 kb 3' to the poly(A) addition site; developmental silencing

Fig. 2. Localization of a developmental-silencing element 3' of the h ζ -globin gene. (A) Structure of three h ζ transgenes with truncations of the 3'-flanking region. A 4.4 kb *Eco*RI genomic fragment is shown. This fragment contains the full-length h ζ -globin gene as well as two head-to-head *Alu* repeats are represented as open boxes. The predicted transcriptional orientations of the *Alu* elements are indicated by arrows. A 6.5 kb DNA cassette containing all hypersensitive sites (HS) (vertical arrows) of the β -LCR is shown to the left of the cluster. The β -LCR cassette was ligated to each gene prior to microinjection. Nomenclature of the genes corresponds to the restriction site at the 3' terminus (*Dra*I, *Bst*1107I or *Nco*I site). (B) RNase protection assay of embryonic and fetal RNA samples. Results from three transgenic littermates of the indicated lines are shown for each construct. (C) Expression of ζ *Nco*, ζ *Bst* and ζ *Dra* transgenes. The levels of transgene expression from six ζ *Nco* lines, four ζ *Bst* and two ζ *Dra* lines are shown. Each value represents the average of at least two determinations carried out on tissues from three or more mice at each time-point (as described for Figure 1).

of transgenes lacking this 108 bp sequence was significantly blunted.

Trans-acting factors present in the nuclei of fetal/adult but not embryonic erythroid cell lines specifically recognized a 24 bp subsegment (SE24) of the putative silencing region

To identify nuclear proteins that might interact with the putative silencing element, the 108 bp DNA fragment (SE108) defined by the transgenic studies was divided

into four overlapping segments for electrophoretic mobility-shift assay (EMSA) (Figure 4A, top). Nuclear extracts were harvested from erythroid cell lines with an embryonic (K562; Lozzio and Lozzio, 1975), fetal/adult (HEL; Martin and Papayannopoulou, 1982) or adult (MEL; Friend *et al.*, 1971) globin expression phenotypes. The first three segments (A–C) failed to form a complex with any of these three nuclear extracts (data not shown). In contrast, the 3'-most fragment (SE24) assembled a DNA–protein complex when incubated with equal quantities of nuclear extracts

Fig. 3. Sublocalization of the h ζ -globin 3'-silencing element to a 108 bp sequence. (A) Diagram of the 408 bp *Nco*I–*Bst*1107I sequence 3' to the h ζ -globin gene. This region, which contains the silencer activity (Figure 2), encompasses an *Alu* repeat that is situated in the opposite transcriptional orientation to the upstream h ζ -globin gene. The *Alu* promoter elements (A and B boxes), the terminal direct repeats (DR) and the *Alu* termination signal (TTT) are each indicated. Shown below are three h ζ transgenes which terminate at 1.1, 1.2 or 1.3 kb 3' to the *Nco*I site. The 108 bp region between the 3' termini of the ζ 1.1 and ζ 1.2 transgenes, containing the putative silencing activity, is boxed (Silencer). (B) RNase protection assay of embryonic and fetal RNA samples from littermates of the indicated representative mouse lines (as described in legend to Figure 1). (C) Expression of ζ 1.1, ζ 1.2 and ζ 1.3 transgenes at two developmental time-points (day 9.5 and day 16.5) (as described in Figure 1). Results from four or five independent transgenic lines are shown for each construct.

from MEL and HEL, but not embryonic K562 cells (Figure 4A, left panel). Competition was observed with the SE24 oligo as well as full-length SE108, but not by a non-specific (NS) probe (Figure 4A, right panels). To evaluate the quality of the nuclear extracts, all three nuclear extracts were shown to form equally strong complexes on a probe containing the binding site for a ubiquitous DNA binding protein (SP1; data not shown). The selective formation of complexes on the SE24 fragment with nuclear extracts isolated from cells with fetal and adult globin gene expression phenotypes suggested that such complexes may

be involved in developmental silencing of the embryonic ζ -globin gene.

SE24 contains an NF- κ B motif and binds NF- κ B in vitro

Sequence analysis revealed that the SE24 contained a canonical NF- κ B binding site (Figure 4B, bottom). NF- κ B has been implicated in the control of a wide variety of gene expression pathways and has been specifically associated with selective inhibition of body patterning pathways in *Drosophila*. To evaluate NF- κ B binding to

Fig. 4. A subsegment of the developmental silencer region bound a p50 NF- κ B complex. (A) A DNA fragment within the putative silencing region specifically interacted with nuclear proteins present in erythroid cells with a fetal/adult globin expression profile but not in embryonic cells stably expressing the h ζ -globin gene. The 108 bp silencing segment (SE108), divided into four overlapping subsegments [A, B, C and D (SE24)], is shown at the top. Below are the electrophoretic mobility gel shift analyses of SE24. The first three segments were inactive in the EMSA analysis. The 32 P-labeled SE24 probe was incubated with nuclear extracts from the indicated cell lines and the resultant products were analyzed by EMSA. A complex formed between the SE24 probe and the MEL and HEL extracts, but not with the K562 extract, is indicated by the arrow. Competition studies were carried out using 200-fold excess of unlabeled SE24 probe, the encompassing SE108 segment [see (A)], or an unrelated oligonucleotide (see Materials and methods) further demonstrating the sequence specificity of the complex formation. (B) The SE24 complex assembled at an NF- κ B motif. The sequences of SE24, SE24m and the consensus (human immunoglobulin κ chain enhancer) NF- κ B binding motif are shown. SE24m is identical to SE24 with the exception of a GG $\rightarrow$ CC substitution within the NF- κ B binding motif (underlined). 32 P-labeled SE24 was incubated with MEL nuclear extract. The complex that was formed (Complex; first lane) was competed by the addition of unlabeled SE24 and NF- κ B but not the mutant SE24m. The SE24m was also unable to directly assemble a complex (right lane). (C) The SE24 complex was recognized by an antibody to NF- κ B p50 and p65 subunits. 32 P-labeled SE24 was incubated with HEL cell nuclear extracts in the presence of antibodies specific to each of the noted NF- κ B subunits: p50, p65 (RelA), p52, p68 (RelB) and p75 (c-Rel). The left panel shows gel mobility shift analysis without or with NF- κ B antibodies. The lower arrow indicates the position of the native SE24 complex and the upper arrows indicate the supershifted complexes. In the right panel 32 P-labeled SE24 was incubated with the indicated nuclear extracts or with human recombinant protein p50. The complexes formed with the recombinant p50 (p50 homodimer) were distinct from the native complex.

this site, a two-base substitution of GG for CC at the highly conserved second and third positions in the NF- κ B consensus sequence was introduced into SE24 sequence. This substitution would be expected to destroy NF- κ B binding (SE24m mutation; Figure 4B, bottom). When analyzed by EMSA, the complex formed between SE24 and the MEL nuclear extract could be competed by SE24 as well as by the immunoglobulin κ chain NF- κ B enhancer element, but not by SE24m (Figure 4B, left panel). Moreover, SE24m was unable to directly assemble the SE24 complex in MEL nuclear extracts (Figure 4B, right panel). These results indicated that one or more factors bound specifically to the NF- κ B motif in the SE24 subsegment of the SE108 silencer domain.

The SE24 complex is recognized by antibodies to NF- κ B p50 and p65

There are five structurally related members of the mammalian NF- κ B/Rel protein family: p50, p52, RelA (p65), RelB (p68) and c-Rel (p75) (Baeuerle and Baltimore, 1996; Baldwin, 1996). These proteins share an N-terminal Rel-homology domain that mediates binding to their cognate DNA sites as hetero- and/or homodimers. EMSA supershift assays were carried out to determine whether any of these NF- κ B subunits were involved in formation of the SE24 complex (Figure 4C, left panel). Antibodies to p50 and p65 were able to recognize and supershift this complex: the supershift by the antibody to p50 was quantitative while that by the antibody to p65 was less pronounced. This complex was not supershifted with antibodies to any of the other three NF- κ B/Rel family members (although convincing positive controls for these antibodies could not be generated). To determine whether p50 binds to SE24 as a homodimer or as a component of a heterodimer (Fujita *et al.*, 1992), SE24 was incubated with recombinant human p50 to assemble a homodimer complex. The recombinant p50 contains no additional sequences and should be identical to the unmodified native protein (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Schmid *et al.*, 1991). The result showed that the SE24 complexes formed with the native nuclear extract and with the recombinant p50 migrated at distinct positions (Figure 4C, right panel).

Mutation of the NF- κ B consensus sequence 3' to the h ζ -globin gene disrupted developmental silencing

The deletion mapping, developmental studies and EMSA analyses detailed above suggest that the NF- κ B motif 3' to the h ζ -globin gene is directly involved in its developmental silencing. To test this model, we introduced the same two-base substitution (GG for CC) at the NF- κ B consensus sequence of ζ 1.2 transgene that was shown to destroy complex assembly *in vitro* (see Figure 4B, bottom). The mutant transgene (ζ 1.2m; Figure 5A) was introduced into the mouse genome. In contrast to the full silencing of the wild-type ζ 1.2 transgene (Figure 3), introduction of the two-base substitution at the NF- κ B element substantially blunted the developmental control *in vivo* (Figure 5). An RNase protection assay from a representative mouse line demonstrated continued expression of the transgene in day 16.5 fetal livers (Figure 5B). Quantitative determinations from four ζ 1.2m lines demonstrated that

Fig. 5. Interruption of the SE24 NF- κ B motif blunts developmental silencing of the h ζ transgene *in vivo*. (A) Diagram of the ζ 1.2m transgene. The CC→GG substitution that destroyed the NF- κ B site in the ζ 1.2 is indicated. (B) RNase protection assay of day 9.5 embryonic yolk sac and day 16.5 fetal liver RNA samples from littermates of a representative line. (C) Expression of four individual ζ 1.2m transgenic lines at two developmental time-points demonstrating blunted silencing activity secondary to the GG→CC mutation at the NF- κ B site.

developmental silencing was blunted by the NF- κ B mutation; the ζ 1.2m transgene exhibited a mean 16-fold decrease in mRNA concentration from day 9.5 to day 16.5 (Figure 5C). This loss of silencing activity is indistinguishable from that seen when the entire 108 bp silencer element is deleted (ζ , ζ Nco and ζ 1.1) and was clearly blunted when compared with that of the wild-type ζ 1.2 transgene (90-fold decrease; Figure 3). These data confirmed a role for the 3'-flanking NF- κ B binding site in the developmental silencing of the h ζ -globin gene.

Discussion

Developmental switching within the globin gene cluster is a highly conserved program (Goodman *et al.*, 1987). In mammalian species the embryonic globin genes are silenced as erythropoiesis switches to the definitive lineage. This definitive erythroblast series, which produces smaller, non-nucleated erythrocytes, is initially established in the fetal liver and subsequently shifts to the bone marrow around the time of birth. Thus, the initial event in globin switching is silencing of the embryonic genes. Whereas a variety of *cis*-acting elements and *trans*-factors, both general and erythroid specific, have been studied in conjunction with activation and enhancement of globin gene expression (Crossley and Orkin, 1993; Miller and Bieker, 1993; Raich and Romeo, 1993), the determinants of globin gene silencing are less well defined. Study of developmental silencing of the embryonic ϵ -globin in the h β -globin gene cluster revealed a silencer 5' to the promoter (Cao *et al.*, 1989; Raich *et al.*, 1992). This silencer element is able to bind either the erythroid-specific transcription factor GATA-1 or the ubiquitous transcription factor YY1; binding of YY1 results in developmental silencing of the ϵ -globin gene (Raich *et al.*,

1995). Multiple silencer elements have been identified for the chicken embryonic ρ -globin gene, distributed in introns as well as in the proximal 5' and 3' flanking regions, but definition of the corresponding *cis* and *trans* determinants have not been reported (Wandersee *et al.*, 1996). Thus, the full complement of components and mechanisms involved in silencing of the embryonic globin genes remains to be explored.

Our previous studies demonstrated that silencing of the human embryonic ζ -globin gene is controlled by negative regulatory elements within 2.1 kb of the 3'-flanking region (Liebhaber *et al.*, 1996). The α - and ζ -globin transgenes used in those studies were linked to the β -LCR to facilitate their expression. To rule out the possibility that the observed developmental profiles might have been influenced by the use of a heterologous (β)-LCR we confirmed our observation of gene-autonomous h ζ -globin gene silencing and the dependence of full silencing on an element(s) in the 3'-flanking region in a setting in which the ζ -globin gene was linked to the corresponding α HS-40 (Figure 1).

Successive deletions in the 3'-flanking region of the ζ -globin gene were performed to map the silencing element. Full developmental silencing activity was localized 1.2 kb 3' to the gene. All five h ζ -globin transgenes (16 independent lines) containing this 108 bp (SE108) segment (ζ *Eco*, ζ *Bst*, ζ *Dra*, ζ 1.2 and ζ 1.3) were appropriately silenced between day 9.5 and day 16.5 (84-, 45-, 77-, 90- and 90-fold silencing, respectively; Figures 2 and 3). In contrast, the three h ζ -globin transgenes lacking the SE108 region (ζ , ζ *Nco* and ζ 1.1) exhibited a blunted decrease in mRNA levels (15-, 11- and 10-fold, respectively) and remained transcriptionally active in fetal erythroid cells (total of 15 independent lines; Figures 1 and 2). These developmental patterns were independent of absolute levels of transgene expression or transgene copy number. These results localized the 3'-silencer element to a 108 bp sequence (SE108) within the original 2.1 kb 3'-flanking region.

The SE108 region was surveyed for binding by nuclear factors that might correlate with its silencing function. EMSA identified a 24 bp subsegment of this region that bound nuclear factors present in erythroid cell lines representing fetal/adult but not embryonic stages (Figure 4A). The source of the embryonic nuclear extract was a cell line expressing ζ -globin and thus assumed to be lacking effective silencing factors. The use of this line circumvented technical problems entailed in analyzing the mixed cell population in primary yolk sac. In addition, our previous studies have revealed that ζ -globin transgene silencing is initiated soon after transcriptional activation and is already appreciable in the yolk sac between days 8.5 and 9.5 (Liebhaber *et al.*, 1996). Consistent with these data we have found that nuclear extracts from the yolk sac contain p50 and bind the SE24 element much the same as extracts from fetal liver (data not shown). Thus, the K562 ζ globin-expressing cell line appeared to be a more reliable source of embryonic nuclear extract that would be lacking silencer factors.

A sequence scan of the SE24 silencer subsegment revealed several putative factor binding sites, including that of the widely expressed NF- κ B complex. Competition and supershift analysis confirmed *in vitro* assembly of NF- κ B at this site and identified the p50 subunit and to a

lesser extent the p65 subunit as taking part (Figure 4B and C). Although the antibodies to the three other known subunits of NF- κ B did not react with the complex, our inability to demonstrate that each of these antibodies can exert a supershift (data not shown) prevents us from firmly ruling out their presence. The quantitative supershift by anti-p50 implied that all the SE24 complexes contained p50. The SE24 complex could therefore have been composed of p50 homodimers, or heterodimers of p50 with a second protein. The distinct migration of the SE24 complex from that formed between the SE24 probe and recombinant p50 argued against substantial p50 homodimerization at this site. The low-level supershift with the p65 antibody suggested that at least a subset of the complexes may contain p65 subunits. Thus, the full composition of the complex formed at the SE24 segment could not be fully determined and might be constituted by heterodimerization between p50 and p65 and/or an as yet undefined partner(s).

The relevance of the NF- κ B site in the SE108 region to ζ -globin gene silencing activity was directly tested *in vivo*. The same GG $\rightarrow$ CC base substitution that interfered with complex formation at the NF- κ B motif *in vitro* also disrupted silencing of the h ζ -globin transgene *in vivo* (Figure 5). The 90-fold silencing mediated by SE108 element in the ζ 1.2 transgene was decreased to 16-fold by this mutation. This residual level of silencing was the same as the 10- to 15-fold decrease seen with 3'-flanking region deletions that removed the SE108 region (10- to 15-fold; Figures 1–3; Liebhaber *et al.*, 1996). Thus the functional importance of the 3'-flanking region element in ζ -globin gene silencing was confirmed.

Since the silencing element 3' to the h ζ -globin gene was studied in the context of the developing mouse embryo it was of interest to ask whether similar elements existed for the endogenous murine ζ -globin gene or for the developmentally co-regulated ϵ -globin gene. A sequence search identified an NF- κ B motif 0.9 kb 3' to the m ζ -globin gene. This element assembled a complex that migrated at the same position as the corresponding human (SE24) complex. The two elements effectively cross-competed and were competed by the consensus NF- κ B motif for protein binding (data not shown). A similar NF- κ B binding motif was also identified within the proximal 3'-flanking sequence of the human embryonic ϵ -globin gene and the mouse embryonic β h0 gene. These observations suggested a generalized role for this determinant in the silencing of embryonic globin genes. However, the activity and function of these additional sites must remain speculative until functionally tested *in vivo*.

NF- κ B was originally identified as an inducible B-lymphocyte-specific factor that bound to a decameric motif present in the intronic κ light chain enhancer (Sen and Baltimore, 1986). Subsequent studies revealed that the expression of a wide spectrum of genes, especially those involved in immune cell activation, inflammation and control of apoptosis, are all enhanced by NF- κ B/Rel proteins through interaction with this κ B-like DNA motif (Liou and Baltimore, 1993). Studies in *Drosophila* suggest that NF- κ B may mediate gene silencing as well. Dorsal, a *Drosophila* homolog of the mammalian NF- κ B/Rel family, negatively regulates expression of maternal effect genes controlling dorsal-ventral pattern formation (Ip *et al.*, 1991; Jiang *et al.*, 1991) This precedent in

Drosophila suggests that similar negative activities may be defined for NF- κ B in mammalian embryonic development. One such activity may be silencing of the ζ -globin gene as presently described. While 'knockout' mice individually null for each of the five murine NF- κ B/Rel subunits fail to show defects in embryonic development there may be a high level of redundancy in this system (Beg *et al.*, 1995; Kontgen *et al.*, 1995; Sha *et al.*, 1995; Weih *et al.*, 1995; Franzoso *et al.*, 1997; Caamano *et al.*, 1998). A specific redundancy in the activities of NF- κ B p50 and p52 is supported by the identification of developmental defects in the differentiation of osteoclasts and B-cells in p50/p52 double knockout, but not single knockout mice (Franzoso *et al.*, 1997; Iotsova *et al.*, 1997). Studies of ζ -globin gene silencing in such knockout models will need to be considered in light of the complexity and redundancy of the NF- κ B system as well as after a more detailed characterization of the full complement of proteins involved in the SE24 complex.

The silencing activity to the 108 bp region between the *Nco*I and *Bst*1107I sites mapped within the AT-rich region of an Alu element. The antisense transcriptional orientation of this element (relative to that of the ζ -globin gene) initially raised the mechanistic possibility that the Alu element repressed h ζ -globin gene expression by transcriptional interference. However, multiple assays failed to detect Alu transcripts in the erythroid cells of the ζ *Bst* mice (data not shown). Furthermore, the h ζ -globin transgene still exhibited proper developmental regulation in the ζ 1.2 mice in which the RNA pol III promoter A box and B box of the Alu was deleted (Figure 3A). These results made it highly unlikely that Alu transcription per se was responsible for the developmental silencing of the h ζ -globin gene. It is of possible interest to note, however, that the identified NF- κ B motif and surrounding AT-rich region is a highly conserved feature of Alu elements (Jurka and Milosavljevic, 1991). As the human haploid genome contains $\sim 3 \times 10^5$ Alu elements, many of which would contain this element, the possibility must be considered that this region may mediate developmental control over other genes as well.

Developmental silencing of the h ζ -globin gene is a complex regulatory process. Our previous data suggested that the promoter, transcribed region and 3' flanking sequences all contribute to this process via distinct transcriptional and post-transcriptional mechanisms (Liebhaber *et al.*, 1996; Russell *et al.*, 1998). When focusing on the silencing that occurs during the yolk sac to fetal liver transition, the transcribed region mediates ~ 6 -fold silencing activity and the 3' flanking region (in conjunction with its promoter) mediates ~ 9 -fold silencing activity (Liebhaber *et al.*, 1996). The combined silencing activity of these two regions in the day 9.5 to day 16.5 developmental window is 50-fold or greater. The silencing activity of the 3'-flanking region is dependent on a specific interaction with the ζ -globin gene promoter as this region fails to silence a chimeric transgene driven by the α -globin promoter (Liebhaber *et al.*, 1996). This suggests a long-distance interaction between the proximal promoter and the SE108 element. Such an interaction might be mediated by multi-protein interactions. In this context it is of interest to note that NF- κ B can interact cooperatively with a number of transcription factors. Synergistic activa-

tion of NF- κ B with Sp1 transcription factor induces transcription of the HIV-1 LTR, and the spatial arrangement of the two respective binding sites is required for this synergy (Perkins *et al.*, 1993). It has been reported that the bZIP domain of c-Jun, c-Fos and several C/EBP proteins interact with the Rel-homology domain of NF- κ B (Matsusaka *et al.*, 1993; Stein *et al.*, 1993a,b). There is evidence that c-Rel is able to associate with the TATA-binding protein (TBP) of the TFIID complex (Kerr *et al.*, 1993). Thus NF- κ B may interact with proteins important for both enhancer and basal promoter function. Such interactions may result in both positive and/or negative effects on gene expression. For example, *Drosophila* Dorsal appears to be a transcriptional activator for genes such as *twist* (*twi*) and *snail* (*sna*) and is converted into a repressor for genes such as *decapentaplegic* (*dpp*) and *zerknüllt* (*zen*) by interacting with corepressor partners. Dorsal-co-repressor complexes exert their silencing activity over long distances. Further analysis of the complex that assembles at the 3'-flanking NF- κ B site may reveal how it communicates with the minimal promoter to mediate developmental silencing.

In addition to its intrinsic interest, delineation of the mechanisms and elements involved in silencing of the embryonic globin genes has potential medical relevance. The loss or dysfunction of the adult α - or β -globin genes, resulting in α - and β -thalassemia, respectively, constitute two of the most common and severely debilitating genetic diseases worldwide (Modell and Bulyzhenkov, 1988). The vast majority of individuals with α - and β -thalassemia have physically intact but functionally silenced embryonic globin genes. It has recently been demonstrated that forced expression of the human embryonic h ζ - and h ϵ -transgenes can reverse lethality in mice homozygous for deletion of α - or β -globin genes, respectively (Russell and Liebhaber, 1998). Thus a full understanding of the mechanism(s) that underlie the silencing of the human embryonic globin genes may point to novel approaches to block their silencing *in utero* or to derepress the already silenced genes in adults with a consequent amelioration of thalassemia.

Materials and methods

Transgene construction

The ζ construct contains the micro β -LCR placed 5' to a 2.3 kb h ζ -globin gene extending from the *Eco*RI site of 559 bp 5' to the cap site to the *Bam*HI site of 106 bp 3' to the poly(A) addition site. The human micro β -LCR, a 6.5 kb *Sst*II fragment isolated from p1417, contains all of the β -LCR DNase I hypersensitive sites (Talbot *et al.*, 1989). To generate the ζ *Nco*, ζ *Bst* and ζ *Dra* deletions, the 1.9 kb *Bam*HI fragment encompassing 3'-flanking sequence of the h ζ -globin gene was isolated, its *Bam*HI sites were converted to *Eco*RI ends by blunting and linker ligation, and the resultant fragment was subcloned into the *Eco*RI site of psp72 vector. This plasmid was then individually digested with *Nco*I, *Bst*1107I or *Dra*I, the free ends converted to *Eco*RI by blunting and *Eco*RI linker ligation, and the resulting *Eco*RI fragments were subcloned into the 3' *Eco*RI site in the ζ construct. The native orientation was identified by restriction analysis.

The ζ 1.1, ζ 1.2, ζ 1.2m and ζ 1.3 deletion transgenes were derived from ζ *Nco* construct. The four respective 3'-extension fragments, generated by PCR using the ζ *Bst* gene as template, were ligated at the *Nco*I site in the native orientation, and were confirmed by sequencing (primer sequences available on request). The 3' primer for ζ 1.2m contained the inactivating GG $\rightarrow$ CC substitution within the NF- κ B consensus sequence.

The h α -globin locus major regulatory element (HS-40; Higgs *et al.*, 1990) was cloned as a 1.4 kb *Eco*RI-*Hind*III fragment from P1 clone #859 (Genome System). HS-40 ζ and HS-40 ζ *Eco* were generated by

ligating HS-40 to the 2.3 kb *EcoRI*–*Bam*HI or 4.4 kb *EcoRI* genomic DNA fragments containing the h ζ -globin gene. The structures of all constructs were verified by restriction analysis and by sequencing PCR amplified segments.

Transgenic mice

Each gene was released from the carrying vector (*EcoRV*–*Sal*I double digestion), separated from the vector on a 0.8% (w/v) agarose gel, and purified by glass bead extraction and Elutip filtration. Transgenic mice were generated as described previously (Liebhaber *et al.*, 1996). Transgenic founders were identified by dot blot analysis of tail DNA and verified on Southern blot analysis with a ³²P-labeled human ζ -globin gene promoter as probe (663 bp *EcoRI*–*Bgl*II fragment). The structure and copy number of the transgene in each line were determined by Southern blot of tail DNA from a positive F1 mouse (Liebhaber *et al.*, 1996). A table detailing the designations of all transgenic lines, their transgene copy number, level of transgene mRNA expression and expression per transgene copy will be supplied upon request.

Quantitative analysis of mRNA

A positive F1 male from each line was mated with non-transgenic CD-1 females. Day 9.5 post-coitum (p.c.) embryonic yolk sacs, and day 14.5 and day 16.5 fetal livers were harvested for developmental studies. The morning when a vaginal copulation plug was observed was day 0.5. The tissue samples were screened for transgene-positivity by DNA dot blot analysis. Total RNA was isolated from transgenic tissues as described by Chomczynski and Sacchi (1987). The mRNA level in each sample was measured by the quantitative RNase protection assay using a series of probes specific for each of the respective mRNAs as detailed previously (Liebhaber *et al.*, 1996). The protected fragments are 150 bp for the h ζ -globin transgene, 179 bp for the endogenous m α -globin gene and 193 bp for the endogenous m ζ -globin gene, respectively. Two of the HS-40 ζ lines were not used due to very low levels of expression at day 9.5. To maximize detection of trace levels of h ζ -globin mRNA expression in the liver, the specific activity of the probes for the h ζ -globin mRNA was adjusted to be 10-fold greater than that for the other two mRNAs. Radioactive signals on analytic gels were quantified by PhosphorImager (Molecular Dynamics Storm 840) and normalized to the probe-specific activity. The level of transgene expression was calculated as a percentage of the sum expression of endogenous m α - and m ζ -globin gene and corrected by the relative specific activities of the probes. The calculation formula is:

$$\text{h}\zeta\text{-globin transgene expression (\%)} = \frac{(\text{h}\zeta \text{ mRNA})}{(\text{m}\alpha + \text{m}\zeta \text{ mRNA})} \times 100.$$

Cell culture and preparation of nuclear extracts

Mouse erythroleukemia (MEL) and human erythroleukemia (HEL and K562) cells (both uninduced) were grown in RPMI 1640 medium supplemented with 10% (w/v) fetal bovine serum, 100 μ g of streptomycin and 100 U/ml of penicillin. By RNase protection assay h ζ -globin mRNA was abundant in K562 and was undetectable in the MEL and HEL cell lines (data not shown).

The nuclear extracts were prepared as described (Dyer and Herzog, 1995) with slight modification. Briefly, 1×10^8 cells were harvested at exponential-growth phase, washed three times with phosphate-buffered saline (PBS) and transferred to a fresh Eppendorf tube. The cells were resuspended in 1 ml of sucrose buffer [0.32 M sucrose, 3 mM CaCl₂, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris–HCl pH 8.0, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% (v/v) NP-40] by gentle pipetting on ice for 5 min. The nuclei were microcentrifuged at 1000 r.p.m., 4°C for 5 min, washed twice with sucrose buffer lacking NP-40 and resuspended in 80 μ l of low-salt buffer (20 mM HEPES pH 7.9, 25% (v/v) glycerol, 1.5 mM MgCl₂, 0.02 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) by gentle pipetting. To extract nuclei, 0.25 vol of high-salt buffer [20 mM HEPES pH 7.9, 25% (v/v) glycerol, 1.5 mM MgCl₂, 0.8 M KCl, 0.2 mM EDTA, 1% (v/v) NP-40, 0.5 mM DTT, 0.5 mM PMSF] was added to 0.25 of the low-salt buffer volume, mixed by gentle vortex and then high-salt buffer continued to be added with mixing until 1 vol has been added. The mixture was incubated at 4°C on a rotary platform with gentle mixing for 20 min, and diluted 1:2.5 with diluent solution [25 mM HEPES pH 7.6, 25% (v/v) glycerol, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF] and microcentrifuged at maximum speed for 15 min at 4°C. The supernatant (nuclear extract) was collected and aliquoted into Eppendorf tubes, and stored at –80°C. The protein concentration of

nuclear extracts was estimated by Bio-Rad protein assay (Bio-Rad #500-0006).

Electrophoretic mobility shift assay

The following double-stranded oligonucleotides were used either as probe or as competitors in EMSAs: SE24 [5′-AGTAGAGACGGGGTTTCACCGTGT-3′], SE24m [5′-AGTAGAGACGCCGTTTCACCGTGT-3′], NF- κ B binding sequence from immunoglobulin κ chain enhancer (NF- κ B) [5′-AGCTTCAGAGGGGACTTTCCGAGAGGTCGA-3′], and mouse 3′-conserved sequence (MCS) [5′-GTTAGGGGAAATCCCAT-GGCT-3′].

The 108 bp of silencing element (SE108) was isolated from the corresponding plasmid clone. Each oligonucleotide probe was end-labeled with [γ -³²P]ATP using T4 polynucleotide kinase. A 20 min binding reaction was performed at room temperature; 10 μ g of nuclear extracts were incubated in 20 μ l of reaction mixtures containing 2 μ g of poly(dI–dC), 3 μ g of bovine serum albumin, 5–10% (v/v) glycerol and 2 μ l of 10 $\times$ binding buffer (100 mM Tris–HCl pH 7.5, 500 mM NaCl, 10 mM EDTA and 10 mM DTT) and 0.5 ng oligonucleotide probe. For competition assays, 200-fold molar excess of unlabeled DNA competitor was added prior to ³²P-labeled probe. In antibody supershift experiments, 1–2 μ l of the antibody was preincubated with nuclear extract for 20 min, followed by addition of the ³²P-labeled probe and further incubation for 20 min. Specific antibodies to NF- κ B subunits p50, p52, p65(relA), p68(relB) and p75(c-rel) were obtained from Santa Cruz Biotechnology. Recombinant p50 protein was purchased from Promega Biotech. The protein is a full-length (453 amino acid) human p50 (Kieran *et al.*, 1990). After incubation, the samples were loaded on 4% (v/v) non-denaturing polyacrylamide gel and run in 0.5 $\times$ TBE buffer at 120 V for 3–4 h.

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