CHAPTER 5
THE EFFECTS OF DELETING HS2 OR HS3 FROM THE LCR IN THE CONTEXT OF A 185KB HUMAN β-GLOBIN LOCUS IN SINGLE COPY TRANSGENIC MICE: A REQUIREMENT FOR HS2 IN ε-GLOBIN EXPRESSION AND A GLOBAL ROLE FOR HS3 IN LOCUS ACTIVATION

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Summary

The human β-globin LCR is required for the high level transcriptional activation of the globin genes in the locus. The LCR consists of five DNase I hypersensitive sites which appear to interact together in forming a single functional unit, termed the holocomplex. We have previously deleted individual HS sites from the LCR and analysed the effects of these deletions on the regulation of the complete 70 kb human β-globin locus in transgenic mice. We found that HS1, HS2 and HS3 deletions rendered the locus susceptible to classical position effect variegation and a novel cell timing position effect. We have extended these studies by repeating the deletions of HS2 and HS3 in the context of a 185 kb human β-globin locus PAC. We analysed single copy transgenic mice with defined chromosomal sites of transgene integration. With the exception of one line that exhibits severe position effects particularly in the foetal liver and adult stages, we found that deletion of HS2 results in a more pronounced reduction of ε-globin expression in the embryonic yolk sac, whereas the effects on γ-globin expression in the yolk sac and γ- and β-globin expression in the foetal liver and adult blood stages were milder. These results confirm that HS2 contributes to LCR function and also reveal a potential requirement of HS2 for ε-globin expression. The deletion of HS3 gives rise to severe position effect variegation in the two transgenic lines tested. These results further confirm the key role that HS3 plays in LCR function. Taken together, these results provide additional support for the holocomplex model of LCR function and additional suggest discrete functions of individual HS sites within the holocomplex.

Introduction

The human β-globin locus contains five developmentally regulated genes organised in the order in which they are activated during development, i.e. 5'-$\varepsilon^{G}\gamma^{A}\gamma\delta\beta$-3'. The locus is regulated by the Locus Control Region (LCR) located upstream of the gene domain. Naturally occurring deletions in the locus that give rise to thalassemia, as well as transgenic mouse studies, have suggested that the LCR is a critical element for the long-range chromatin organisation and transcriptional activation of the β-globin locus (Grosveld, 1999). The LCR is comprised of five DNase I hypersensitive sites (denoted HS1-5) spanning a region of approximately 21kb immediately upstream of ε-globin, the 5'-most gene in the locus (Tuan *et al.*, 1985 and Dhar *et al.*, 1990). Highly localised DNase I hypersensitivity in this region underlies a high concentration of binding sites for transcription factors, both haematopoietic-restricted and ubiquitous (Talbot *et al.*, 1990; Philipsen *et al.*, 1990; Pruzina *et al.*, 1991and Zafarana *et al.*, 1995). Several studies have been previously carried out in attempting to delineate the functional properties of individual HS sites and the contribution of each HS site to full LCR function. For example, HS2 was shown to act as a classical enhancer in transient transfection assays as well as in stable transfection assays (Caterina, 1994 and Talbot *et al.*, 1990). By contrast, HS3 and HS4 can activate transcription only in a chromatin context (Philipsen *et al.*, 1990; Pruzina *et al.*, 1991 and Tuan *et al.*, 1992). In transgenic mice, HS2, HS3 and HS4 were each shown to be independently capable of driving high level transcription of linked human β-globin transgenes, though at lower levels than those obtained when using the full LCR (Fraser *et al.*, 1990; Talbot and Grosveld, 1991; Pruzina *et al.*, 1991 and Philipsen *et al.*, 1993). In addition, when individually linked to a cosmid construct bearing the human γ- and β-globin genes, HS2, 3 and 4 were able to activate high levels of transcription with different developmental-stage specificities (Fraser *et al.*, 1993). Finally, when analysed in single-copy transgenic mice, only HS3 was found to be capable of consistently driving high levels of expression that were related to transgene copy-number (Ellis *et al.*, 1996). These observations led to the suggestion that the individual HS sites in the LCR interact with each other to form a holocomplex which acts as a single functional entity in the long-range chromatin organisation and transcriptional activation of the human β-globin locus by the LCR (Fraser *et al.*, 1993). This model gained further support by the demonstration that the LCR interacts with only one gene at a time, as though it presents a single target for interaction, but can activate more than one gene by "flip-flopping" between genes in the locus (Wijgerde *et al.*, 1995).

More recent approaches aimed at gaining insight into the role of each HS site in LCR function have involved the deletion of individual HS sites from the LCR and their analysis in the context of the complete human β-globin locus regulation in transgenic mice (Bungert *et al.*, 1995; Milot *et al.*, 1996; Peterson *et al.*, 1996; Navas *et al.*, 1998; Bungert *et al.*, 1999 and Navas *et al.*, 2001). Our own previous work, involved the deletion of each of HS1, HS2, HS3 and HS4 in the context of two ligated cosmids that together spanned ~70 kb of the human β-globin locus (Milot *et al.*, 1996). With the exception of HS4 which did not affect regulation of the human locus, these deletions rendered expression of the human globin genes sensitive to chromosomal positions effects, where levels of expression for all genes were low and not always directly related to transgene copy number (Milot *et al.*, 1996). These effects were most profound in transgenic mice with pericentromeric integrations of the HS1-, 2- or 3-deleted constructs and were evident in all developmental stages. A closer examination of expression in single erythroid cells, revealed two types of position effects in these mice. Firstly, classical position effect variegation (PEV) was observed in which only a subset of cells (variable between transgenic lines) transcribed the transgene. A second type of position effect was also observed, in which all cells transcribed the transgene but at a lower level per cell. This observation suggested that transcription of the human β-globin genes was taking place in all cells but for a shorter period of time during the cell cycle. This type of position effect had not been previously described and was termed cell timing position effect (CTPE; Milot *et al.*, 1996).

Our results, however, were in contrast with a number of other studies. For instance, deletion of HS2 in the context of a 246 kb human β-globin locus YAC by Peterson *et al.* (1996), showed only a small decrease in the expression of all globin genes at all developmental stages. Bungert *et al*. (1999) reported a severe decrease in expression of all genes when HS2 was deleted in the context of a 155 kb YAC. In addition, deletion of HS3 in the study of Peterson *et al.* (1996) showed a milder effect on the expression of human globin genes, especially in the foetal liver and adult stages, whereas deletion of a smaller HS3 fragment by the same group (Navas *et al.*, 1998) showed a more severe effect on human globin gene expression, particularly in the foetal liver and adult stages. By contrast, Bungert *et al.* (1995) found expression of all human genes to be severely impaired in all developmental stages following deletion of HS3, which is in agreement with our observations. However, in contrast to our observations, Bungert *et al.* (1995) found that the deletion of HS4 also had a severe effect on expression of the human genes. These observations on the effects of the HS4 deletion are also at odds with the findings of Navas *et al.* (2001) who found that a similar deletion of HS4 had a more pronounced effect in the foetal liver and adult stages with a much milder effect in the embryonic stage. The differences between the studies could be due to the use of different constructs, different deletions, different methods of analysis of transgene integrity and globin expression levels, differences in copy-numbers and in integration sites of the transgenes. It should be mentioned that nobody (except our group), did check the integration site of the transgene, which does make a difference in outcome of a study.

In this paper, we repeated the deletions of HS2 and HS3 from the human β-globin LCR. These deletions are identical to the ones carried by Milot *et al.* (1996) but were carried out in the context of a 185 kb human β-globin locus PAC. The main impetus behind this work has been to obtain more transgenic lines with these HS deletions since the observations of Milot *et al.* (1996) raised the intriguing possibility that different types of position effects are associated with the deletion of specific HS sites (e.g. CTPE was observed with the HS3- and HS1- but not with the HS2-deleted mice, raising the potential that it was a HS1- or HS3-specific effect). In addition, in repeating these deletions, we incorporated a number of important differences with the aim of facilitating the interpretation of results. Firstly, the deletions were carried out in the context of a 185 kb human β-globin locus PAC containing much more flanking sequence compared to the 70 kb locus obtained by ligating two cosmids together (Milot *et al.*, 1996), the extra flanking sequence may buffer the transgene from severe chromosomal position effects. These constructs are also structurally closer to the human β-globin locus YACs that have been used in the HS deletion studies by Bungert *et al.* (1995 and 1999), Peterson *et al.* (1996) and Navas *et al.* (1998 and 2001). Secondly, the β-globin locus PAC was fitted with a loxP site upstream for the $^{G}\gamma$ gene so that the effects of the HS deletions could be studied in single copy transgenic animals, generated by crossing multi copy mice with transgenic mice expressing Cre recombinase in the germline. The analysis of HS deletions in single copy mice should remove the

added complication arising from interactions between transgenes in multi copy mice, as has been previously observed (Ellis *et al.*, 1996). Finally, in contrast to the previous studies using globin locus YACs, the chromosomal sites of transgene integration were identified in all transgenic lines generated and analyses of expression were carried out at the single cell level.

Results

Generation of LCR deletion transgenic lines

HS2 and HS3 were deleted from the LCR in the context of a 185 kb PAC containing the entire human β-globin locus with extensive flanking sequence (approximately 50 kb on either side of the locus; A. Imam, personal communication). The deletions were performed by homologous recombination in *E. coli* as described by Imam *et al.* (2000). The human β-globin locus PAC has been previously fitted with a loxP site upstream of the $^{G}\gamma$ gene (Imam *et al.*, 2000). The fragments used to delete the core region of the hypersensitive sites were the same as those previously described by Milot *et al.*, 1996 (Fig. 1A). The targeting fragments, a 2.8 kb HindIII fragment containing the 700 bp core deletion for HS2 and a 4.2 kb HindIII fragment containing the 1.4 kb core deletion for HS3, were cloned into the HindIII site of the pDF25 recombination vector. After homologous recombination, the correct excision events were checked by Southern blot analysis of HindIII digests for both HS2 and HS3 deletions.

In order to ensure that no re-arrangements had occurred in the human β-globin gene locus other than the targeted loxP integration and HS deletions, the wild type and HS-deleted PACs were checked by Southern blotting following homologous recombination and excision, using the LCRε and γγδβ cosmids as probes which, together, cover the ~75 kb human β-globin gene locus and LCR (Fig. 1B). Unrearranged PAC clones for each construct were selected and digested with Not I to release the globin locus insert. The correct size of the released fragment was checked by PFGE and subsequently isolated by salt gradient and prepared for microinjection into the pronuclei of fertilised mouse eggs.

From these microinjections we obtained 12 founders for the wild type β-locus, 8 founders for the HS2 deletion (ΔHS2) and 7 founders for the HS3 deletion (ΔHS3). Genomic DNA from tail biopsies from all of the founder mice was checked for the presence of the PAC transgene, using a probe detecting intron 2 of the β-globin gene at the 3' end of the locus and a probe which detects HS5 at the 5' end of the locus. The integrity of the 70 kb human β-locus and the presence of the HS2 and 3 deletions in the transgenes were checked in two ways after the transmission of the transgene to the F1 generation. Firstly, EcoRI-digested DNA was probed for integrity of the ~75 kb β-globin locus by Southern blotting and probed with the LCRε and γγδβ cosmids, as previously described (Strouboulis *et al.*, 1992). This assay reveals deletions and rearrangements within the 75 kb locus (data not shown). In addition, genomic DNA was digested with a number of additional restriction enzymes that together generate overlapping fragment spanning a region of approximately 100 kb across the β-globin locus and hybridised with different probes. In this way, linkage of the various fragments detected across the human β-globin locus can be demonstrated (data not shown). These assays do not test for integrity of the ~185 kb PAC-derived transgene in regions outside the ~100 kb region covered by the analysis. From these analyses, 9 wild type, 8 ΔHS2 and 6 ΔHS3 founders showed the human β-locus to be intact and were further bred in order to establish transgenic lines. Of those, 8 wild type, 4 ΔHS2 and 4 ΔHS3 founders transmitted the transgene to the F1 generation (Table 1).

For all transgenic lines we determined the chromosomal integration site of the transgene DNA FISH of metaphase spreads. We made a distinction between euchromatic (i.e. any integration other than pericentromeric or telomeric), (peri)-centromeric and (peri)- telomeric integration types. All three types were observed in both wild type and deletion lines (Fig. 2 and Table 1).

Copy numbers for each line were determined using the human β-globin intron 2 probe and a probe against the endogenous mouse carbonic anhydrase II (CA-II) gene as control (see materials and methods). The multiple copy transgenic lines were bred to Zp3-cre transgenic mice to obtain single copy transgenic mice. These mice express the Cre recombinase during oocyte maturation, thus resulting in the recombination to single copy of a loxP-fitted transgene in the eggs of these mice

(Lewandoski *et al.*, 1997). Except for two of the ΔHS3 transgenic lines, all other multi-copy lines could be bred to single copy, as shown by copy number determination. The recombination of these lines to yield single copy transgenes was also checked by comparing transgene expression levels between the multi copy and single copy animals (see below). All single copy lines were again checked for integrity of the human β-globin locus and were found to be intact (data not shown). The fact that we couldn't breed two ΔHS3 lines to single copy could be due to the integrated copies not being in a head-to-tail arrangement which is required for the correct alignment of the LoxP sites for Cre-mediated recombination. Our analyses for integrity of the human β-locus as described above, do not address the orientation of integrated transgene copies relative to each other.

We chose three wild type transgenic single copy lines and all single copy ΔHS2 and ΔHS3 transgenic lines for further analysis.

Expression analysis of the transgenic lines

RNA was isolated from the adult blood of the single copy transgenic lines and their multi copy parent lines and globin gene expression was analysed by S1 nuclease protection. All transgenic lines showed an equivalent fold lower expression of the human β-globin gene in the single copy lines compared to the multiple copy lines (data not shown). This confirms the copy number-dependent expression of the human β-globin gene in our transgenic lines, as has been described previously (Grosveld *et al.*, 1987 and Blom van Assendelft *et al.*, 1989).

To analyse the effects of the HS site deletions on expression of the human globin genes during mouse embryonic development, we isolated yolk sac from 10.5dpc embryos, foetal livers from 14.5dpc embryos and blood from adult mice. Total RNA was isolated from these tissues and human globin gene expression was analysed against that of the endogenous mouse β-like globin genes by S1 nuclease protection assays (Strouboulis *et al.*, 1992).

The three wild type transgenic lines showed similar expression patterns at all time points (Fig. 3 and Fig. 4). The levels and developmental expression patterns obtained with the three wild type lines are comparable to those previously reported for transgenic mice containing cosmid, BAC and YAC based human β-globin locus transgenes (Strouboulis *et al.*, 1992; Gaensler *et al.*, 1993; Peterson *et al.*, 1993; Bungert *et al.*, 1995; Porcu *et al.*, 1997; Kaufman *et al.*, 1999 and Alami *et al.*, 2000). Human globin gene switching also took place in these mice as previously described, although the γ to β switch occurred a bit later than observed with the cosmid based β locus transgenic mice (Strouboulis *et al.*, 1992), but in agreement with the timing reported for YAC transgenic mice (Kaufman *et al.*, 1999; Peterson *et al.*, 1993 and Bungert *et al.*, 1995).

Expression analysis of the ΔHS2 transgenic lines showed different effects for different human globin genes at different developmental stages (Fig. 3, Fig. 4). The most severe effect resulting from the deletion of HS2 was in the expression of human ε-globin in the embryonic yolk sac. Lines 4 and 5 (centromeric and euchromatic integrants, respectively) show an almost complete lack of ε-globin expression with lines 6 and 7 (both telomeric integrants) also showing markedly reduced ε expression (Fig. 3, Fig. 4). Expression of γ-globin in the same stage is also markedly reduced in all ΔHS2 lines, but the effect is milder compared to ε-globin (Fig. 3, Fig. 4). The overall transcriptional output of the human globin genes (ε + γ) compared to total mouse globin output (εy + βH1) is reduced, primarily due to the sharp reduction in human ε expression.

Expression analysis in the foetal liver and adult blood stages, shows γ-globin expression to be only mildly affected by the deletion of HS2 compared to the wild type locus control mice (Fig. 3, Fig. 4). Expression of human β-globin appears to be affected to a greater extent compared to γ expression, but the effects are again more moderate compared to ε-globin expression in the yolk sac (Fig. 3, Fig. 4). Overall transcriptional output of the human globin genes (γ + β) compared to β_{maj} is reduced in the absence of HS2 in the foetal liver and adult blood stages. An exception is line 5 which exhibits severely reduced levels of human γ- and β-globin expression in the foetal liver and adult blood stages (Fig. 3, Fig. 4). Despite its apparent euchromatic integration site, the human globin locus in this line appears to suffer from a severe chromosomal position effect which becomes very evident in the foetal liver and adult stages.

Analysis of the ΔHS3 transgenic lines showed for both lines (lines 8 and 9, euchromatic and pericentromeric integrants, respectively) a severe reduction in expression of all human globin genes at all developmental time points (Fig 3, Fig 4). It is striking that in the adult blood stage there is hardly any human β-globin expression detectable in both lines. The deletion of HS3 therefore leads to severe position effects in these lines, as has been previously observed (Bungert *et al.*, 1995 and Milot *et al.*, 1996 (only observed in some lines)).

Primary transcript in situ hybridisation

The basis of chromosomal position effects becomes evident by assaying for transgene expression at the single cell level (Milot *et al.*, 1996 and McMorrow *et al.*, 2000). To test whether the decrease in human globin gene expression in the HS-deleted mice is reflected in the number of transcribing loci in the nucleus, we carried out primary transcript *in situ* hybridisation on 10.5 day embryonic blood and 14.5 day foetal liver cells from the same samples that were analysed by S1 nuclease protection.

The transcription patterns in 10.5dpc embryonic blood were analysed using gene-specific intron probes for mouse α-, human ε- and human γ-globin genes (Fig. 5A-B, Table 2). In agreement with the S1 nuclease protection data, we find a decrease in the number of human globin gene loci actively transcribing ε-globin (Fig 5A and Table 2). At the same time we observe an almost imperceptible reduction in the number of human globin loci actively transcribing the γ genes, compared to the wild type locus control mice (Fig 5B and Table 2). This is in contrast to the S1 nuclease protection results which indicated a marked reduction in γ-globin expression levels. Taken together, these observations suggest that deletion of HS2 does not appear to affect the number of γ-globin transcribing loci, however, transcriptional output from the γ genes (which cannot be measured in the *in situ* hybridisation assays) appears to be compromised.

For the ΔHS3 lines, we observe an almost complete lack of ε-globin transcription in embryonic blood and a severe reduction in the number of γ-globin transcribing loci (Fig 5B and Table 2) in agreement with the S1 assays.

Primary transcript patterns in 14.5 day foetal livers were analysed using intron probes specific for the mouse α-and human γ-and β-genes. The transcription patterns observed for γ-globin in the ΔHS2 mice are in agreement with the results obtained from the S1 analysis in that the drop in steady-state mRNA levels is accompanied by a decrease in the number of human γ transcribing loci (Table 3). For ΔHS2 line 5 and both ΔHS3 transgenic lines, the severe reduction in human globin gene expression observed in the S1 protection analysis is also evident in the primary transcript *in situ* hybridisation.

In situ mRNA analysis

In order to address the question whether the low expression levels of the human genes in the different deletion lines (line5, 8 and 9) was caused by classical position effect variegation (PEV) (Milot et al., 1996), a reduction of the number of cells expressing the transgene, we performed mRNA *in situ* hybridisation on 14.5dpc foetal liver cells of the same embryos used for S1-nuclease protection assays and primary transcript *in situ* hybridisations. Expressing erythroid cells were visualised with an exon specific oligo for mouse-β_{maj} and cells expressing the transgene were identified with either an exon specific oligo for human-γ or for human-β.

The mRNA *in situ* hybridisation showed for all three deletion lines a clear reduction of number of cells expressing the transgene in comparison to the wild type transgenic lines. We conclude from this that these three deletion lines (one ΔHS2, line 5, and both ΔHS3, line8 and 9) show position effect variegation.

Discussion

In this study we have used a 185 kb PAC containing the human β-globin locus to investigate the influence of the deletion of either HS2 or HS3 of the LCR on the regulation of human β-globin gene locus in transgenic mice. We generated a number of transgenic lines bearing the wild type and the

HS2- and HS3-deleted human globin loci. Expression of human globin genes was analysed in single copy mice with chromosomal integration sites identified. We found that all wild type PAC transgenic lines analysed were regulated similarly to human β-globin locus transgenic mouse models previously generated by a number of groups using ligated cosmids, YACs and BACs (Strouboulis *et al.*, 1992; Peterson *et al.*, 1993; Gaensler *et al.*, 1993; Bungert *et al.*, 1995; Porcu *et al.*, 1997; Kaufman *et al.*, 1999 and Alami *et al.*, 2000).

The effects of deleting HS2 and HS3 on human β-globin locus regulation

We analysed four single copy transgenic mouse lines bearing the HS2 deletion. We found that the gene most severely affected by the HS2 deletion in all four lines was that of human ε-globin in the embryonic yolk sac. The mRNA expression analysis and the primary transcript *in situ* hybridisation data suggested that the effects on ε gene expression were due to a drop in the number of human globin gene loci actively transcribing ε-globin. The decrease of ε expression in all ΔHS2 lines indicates that interaction of HS2 with the ε-promoter is of importance for normal expression of the gene. These observations may be related to recent work which has shown that the presence of HS2 is required for the specific histone H3 hyperacetylation of a TATA box-proximal nucleosome in the ε-globin promoter in episomally maintained minichromosomes (Gui and Dean, 2001). On the basis of these observations it was suggested that HS2 is responsible for the recruitment of a specific acetylase activity responsible for the high-level modification of histone H3 at the proximal promoter in ε-globin activation (Gui and Dean, 2001).

By contrast, γ-globin gene expression in the embryonic yolk sac is only moderately affected by the HS2 deletion. Interestingly, the reduction in γ-globin mRNA levels does not appear to be reflected to the same extent in the number of γ-globin transcribing gene loci. This suggests that while the frequency and/or stability of interaction of the LCR with the γ-globin genes may not be significantly affected by the HS2 deletion, the transcriptional output of the genes is reduced. The same does not appear to be true for γ-globin expression in the foetal liver stage. In this case the (moderate) reduction in γ gene mRNA levels is accompanied by a reduction in the number of human globin loci actively transcribing γ-globin. Interestingly, β-globin gene expression in the foetal liver and adult blood appears to behave in a manner similar to that of γ-globin in the embryonic yolk sac, i.e. the reduction in β-globin mRNA levels is not accompanied by a similar reduction in the number of actively transcribing β gene loci. Take together, these data suggest that HS2 may have more specialised function(s) in the embryonic yolk sac stage, in addition to its more general functions in the transcriptional activation of all genes in the locus. The latter function may be related to the transcriptional enhancer activity previously reported for HS2 in transient transfection assays (e.g. Caterina *et al.*, 1994).

Our observations described here on the HS2 and HS3 deletions, present differences as well as similarities to previous reports on the effects of HS site deletions on the regulation of the human β-globin locus in transgenic mice (Bungert *et al.*, 1995; Milot *et al.*, 1996; Peterson *et al.*, 1996; Navas *et al.*, 1998; Bungert *et al.*, 1999 and Navas *et al.*, 2001). It is difficult to directly compare and reconcile these differences since there are significant variations in the constructs employed and in the ways the deletions were made, the transgenes were analysed and expression patterns determined. What is perhaps more pertinent is the comparison of the HS2 and HS3 deletions presented here to those carried out by Milot *et al.* (1996) in the context of the ~70 kb locus obtained through the ligation of two cosmids. In both studies the deletions for HS2 and HS3 were identical. However, in the present study the β-locus was part of a 185 kb transgene, thus carrying extensive additional flanking sequence (~50 kb on either side of the β-globin locus) compared to the 70 kb locus transgene used by Milot *et al.* In addition, our present analysis on the HS2 and HS3 deletions was restricted to single copy mice. The study by Milot *et al.* (1996) included two single copy lines for the HS2 deletion and only multi copies for the HS3 deletion.

The most significant difference observed between our study and that of Milot *et al.* was related to the effects of the HS2 deletion. Milot *et al.* found that deleting HS2 had a severe effect on the expression of all genes, with the locus being subject to chromosomal position effects. By contrast, we find that the deletion of HS2 has a more severe effect on expression of ε-globin, with γ- and β-globin

gene expression being only mildly affected, with the exception of ΔHS2 line 5 which shows a severe position effect in the foetal liver and adult stages.

By contrast, our study agrees with that of Milot *et al.* on the severe effects that deleting HS3 exerts on the regulation of the locus. Similar effects were also observed in the context of a YAC by Bungert *et al.* (1995). These observations suggest that the presence of extra flanking sequences cannot provide additional function(s) that can protect the locus from chromosomal position effects or that can partly substitute for HS3 activity. We did not, however, observe any CTPE phenomena in our new ΔHS3 lines. This is probably due to the fact that only two lines were analysed, but could also be because the additional flanking sequence may protect against this type of position effect.

The analysis of the effects of deleting HS2 and HS3 on the regulation of the human β-globin in transgenic mice provides further support for the holocomplex model, with different contributions of each HS site towards full LCR function. Our analysis presented here suggests a functional specialization of HS2 in ε-globin regulation, as well as a more general transcriptional activation function. The effects of deleting of HS3 are in agreement with its suggested role as a key element in the long-range chromatin organization in the globin locus.

Materials and Methods

Human β-globin locus PAC constructs and transgenic mice

Hypersensitive site two and three of the locus control region of the human β-globin locus were deleted in the PAC 148loxp containing the human β-globin locus (70 kb) plus additional 3' and 5' sequences (115 kb), using the homologous recombination protocol as described by Imam *et al.*, 2000. The homologous recombination for the introduction of the loxP site in the locus has been described in Chapter 4. The deletions span a region of 742 bp for HS2 and 1384 bp for HS3 and were previously described in Milot *et al.* (1996). A 2.8 kb HindIII fragment and a 4.2 kb HindIII fragment containing the HS2 and HS3 core deletions respectively, where cloned in the HindIII site of the pDF25 recombination vector. After recombination, the ΔHS2 and ΔHS3 PACs were checked for integrity by Southern blot using two cosmid probes, one containing the LCRε region and a second containing the γγδβ region (Strouboulis *et al.*, 1992). The 185 kb PAC insert was isolated by Not I digestion and purified from vector sequences by salt gradient centrifugation, essentially as described by Dillon and Grosveld (1993). Briefly, the digested PAC was layered on top of a 5-25% NaCl gradient and centrifuged at 40,000rpm, room temperature for 50 minutes in a SW41 swing-out rotor. 0.5ml fractions were collected and analysed by agarose gel electrophoresis. Fractions containing only the PAC insert were pooled and dialysed against a large volume of TE (10mM Tris-HCl pH 8.0, 1mM EDTA)/0.1M NaCl for 5 h at 4^0C in UH 100-75 dialysis tubing (Schleicher & Schuell). Dialysis was continued overnight at 4^0C after replacing the buffer. The PAC insert was concentrated by vacuum dialysis and subsequently dialysed against a large volume of microinjection buffer (10mM Tris-HCl pH 7.4, 0.1mM EDTA) containing 0.1M NaCl in order to protect the high molecular weight PAC insert DNA from shearing during microinjection. The purified PAC fragment was checked for DNA integrity and concentration by pulsed field gel electrophoresis in a 1% agarose gel in 0.25XTAE buffer using a Biometra RotaphorType V apparatus, under the following conditions: 8-2sec pulse interval logarithmic ramp, 120-110° rotor angle linear ramp, 200-180volt logarithmic ramp, rotor speed 6 at 13°C for 21hours. The purified insert was injected at approximately 0.5ng/μl into the pronucleus of fertilised eggs of FVB/N mice. The injected eggs were transferred into the oviducts of pseudo-pregnant BCBA foster females as previously described (Kollias *et al.*, 1986).

Transgenic founders were identified via Southern blot analysis using as probes the 970 bp BamHI-EcoRI βIVS2 fragment and a 3.3 kb EcoRI fragment containing HS5. After transmission of the transgene to the F1, the β-globin locus was checked for integrity using the LCRε and γγδβ cosmid probes and specific probes within the LCR and the different genes. The genomic DNA was digested with: EcoRI, BamHI, BglII, HindII, HindIII, EcoRV, SacI, PstI, XhoI-KpnI, XhoI-ApaLI and SacII-KpnI. Southern blots were hybridised with LCRε and γγδβ cosmid probes, HS5 (3.3 kb EcoRI fragment), ε (340 bp BamHi-BspMI fragment), 5' $^{A}\gamma$ (1.7 kb EcoRI-BamHI fragment), β intron II (0.9

kb BamHI-EcoRI fragment) and 5'δ (0.98 kb Xba I-Bgl II fragment). Transgene copy numbers were determined using as probes the βIVS2 fragment and a 0.9 kb Pvu I fragment from the endogenous mouse carbonic anhydrase II (CA-II) gene. The ratios of intensities of the βIVS2 /CA-II bands obtained for the PAC transgenics were compared to those obtained for the single copy human β-globin locus transgenic lines 2 and 72 (Strouboulis *et al.*, 1992). Analysis was performed by PhosphorImager using ImageQuant software (Molecular Dynamics). Multi copy lines were bred to single copy by crossing them with Zp3-Cre transgenic mice expressing the Cre recombinase during oocyte maturation (Lewandoski *et al.*, 1997). Mice obtained from these crosses were checked for copy numbers as above. In addition, comparison of human β-globin expression levels between the parental multi copy lines and the Cre-recombined mice was also carried out by S1 protection analysis in testing for single copy mice. The single copy lines thus obtained, were also checked for integrity of the human β-globin locus as above.

DNA FISH analysis

Peripheral blood cells were cultured for 72 hours in RPMI 1640 medium. Chromosome preparations were made according to standard procedures. FISH was carried out as described by Mulder *et al.* (1995). The specific probe used was the biotin-labelled human β-globin LCR to detect the transgene and was immunochemically detected with fluorescein. Chromosomal DNA was counter-stained with DAPI, which stains centromeric domains more intensely.

RNA fluorescent in situ hybridisations

Mouse 14.5dpc foetal liver and 10.5dpc embryonic blood cells were isolated, fixed and hybridised essentially as described previously (Wijgerde *et al.*, 1995 and van de Corput and Grosveld, 2001). For detection of each globin gene transcript, a mixture of two, three or four different 50-mer oligodeoxynucleotide probes was used. Each oligo probe was labelled with dinitrophenol (DNP), digoxigenin (dig) or biotin (bio) in the middle and at the 3' and 5' ends. Probes for primary transcript and mRNA *in situ* hybridisations were designed from the intron and exon sequences, respectively, of the different globin genes, such that they are at least 25 nucleotides apart. In mRNA *in situ* hybridisations on 14.5 dpc foetal liver cells we used a combination of mouse β_{maj}-DNP and human β-bio and a combination of β_{maj}-DNP and human γ-bio. In the primary transcript *in situ* hybridisations on 14.5 dpc foetal liver cells we used a combination of mouse α-dig and human β-bio. In the primary transcript *in situ* hybridisations on 10.5 dpc embryonic blood we used a combination of mouse α-dig and human γ-bio, a combination of mouse α-dig and human ε-bio. Overnight incubation at 37°C of fixed cells with the oligodeoxynucleotide probe mixtures was followed by detection of the DNP, dig and bio labels with specific antibodies (van de Corput and Grosveld, 2001). Antibody detection was done using a five-layer avidin texas red tree (avidin texas red, goat-α-avidin, avidin texas red, goat-α-avidin and avidin texas red) for bio, a three-layer FITC (green) tree (sheep-α-dig, rabbit-α-sheep fitc, goat-α-rabbit fitc) for dig and a three-layer FITC (green) tree (rat-α-DNP, rabbit-α-rat fitc, goat-α-rabbit fitc) for DNP. Fluorescence was detected by epifluorescence/CCD.

S1 nuclease protection assays

S1 nuclease protection analysis was carried out with total RNA from 10.5dpc embryonic yolk sac, 14.5dpc foetal livers and blood from adult animals. RNA was isolated using the Trizol reagent according to the manufacturer's instructions (Life Technologies). Conditions for S1 nuclease protection assays and polyacrylamide gel electrophoresis were essentially as previously described (Fraser *et al.*, 1990; Kollias *et al.*, 1986; Strouboulis *et al.*, 1992; Milot *et al.,* 1996 and Chapter 4 of this thesis). Specific activities of probes were determined as previously described (Lindenbaum and Grosveld, 1990) and are indicated in the Figure legends. Quantitation was done on a PhosphorImager using the ImageQuant software (Molecular Dynamics).

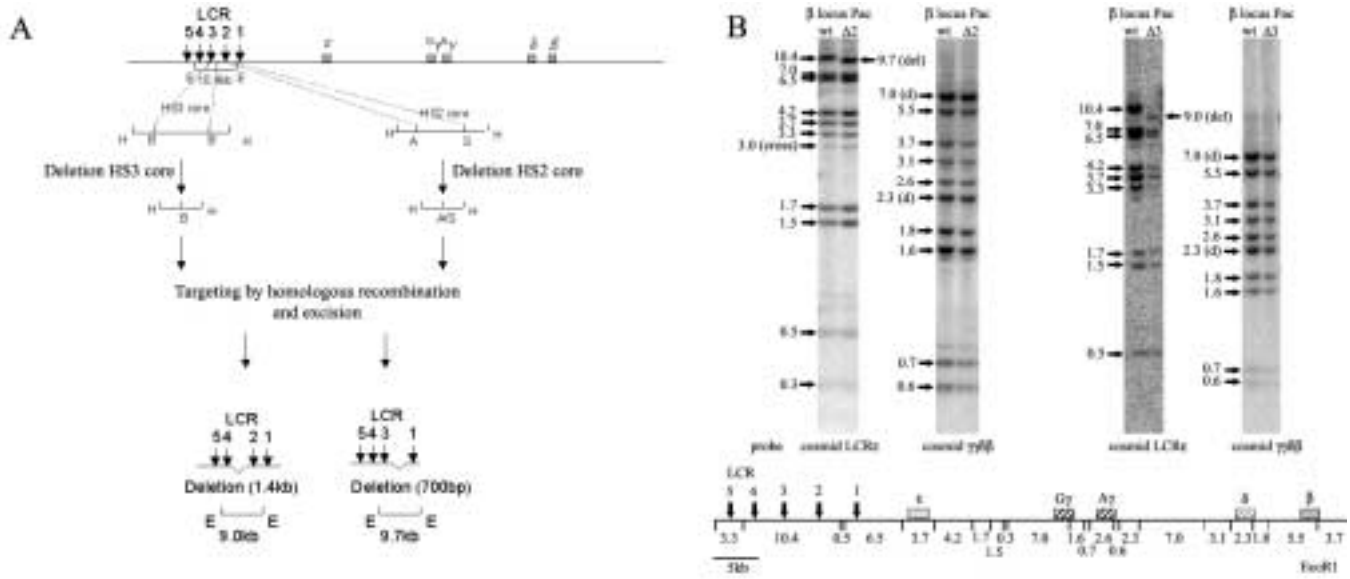

Figure 1*: Construction of the pac148loxp hypersensitive site deletion constructs*—(A): On top a representation of the PAC-insert containing the human β-globin locus. The hypersensitive sites of the LCR are indicated with five black arrows and small boxes represent the globin genes. Homologous recombination followed by temperature sensitive excision (Imam *et al.*, 2000) resulted in deletion of the HS3 and HS2 core regions. (EcoRI fragments as indicated below the LCR). E: EcoRI; B: BamHI; H: HindIII; A: ApaLI and S: SnaBI (B): Mapping of integrity of the human β-locus in the PAC constructs following recombination in E. coli. Southern blots of wt and HS2/3 deleted PAC DNA digested with EcoRI. Bands specific for the human β-locus are detected with LCRε and γγδβ cosmid probes. Arrows indicate the bands present in the EcoRI map of the human β-locus, as shown at the bottom. The diagnostic 9.7 kb fragment arising from the HS2 deletion and the 9.0 kb fragment of the HS3 deletion are indicated to the right of the LCRε panels. Cross: mouse cross-hybridising fragment often observed with the LCRε probe; d: fragments appearing as doublets; del: fragments arising from the HS2 or HS3 deletions.

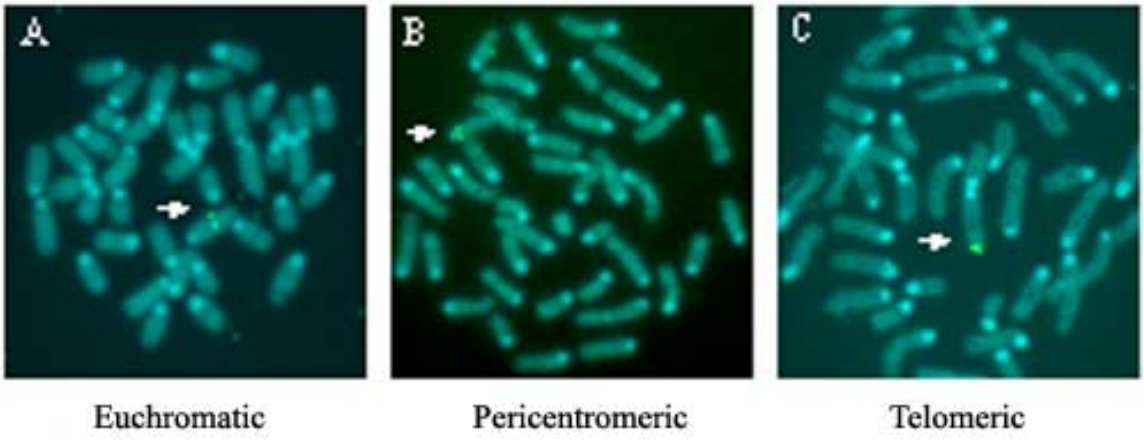

Figure 2*: FISH analysis of metaphase spreads from circulating lymphocytes in the adult blood of transgenic lines*—The human β-globin locus is detected with a dig labelled plasmid containing the LCR (green spot). The chromosomes are visualised with DAPI staining. Arrows indicate site of integration on the chromosome. Three examples are shown. A: euchromatic integration, B: (peri) centromeric integration, C: telomeric integration. All three types were observed in both wild type and deletion lines.

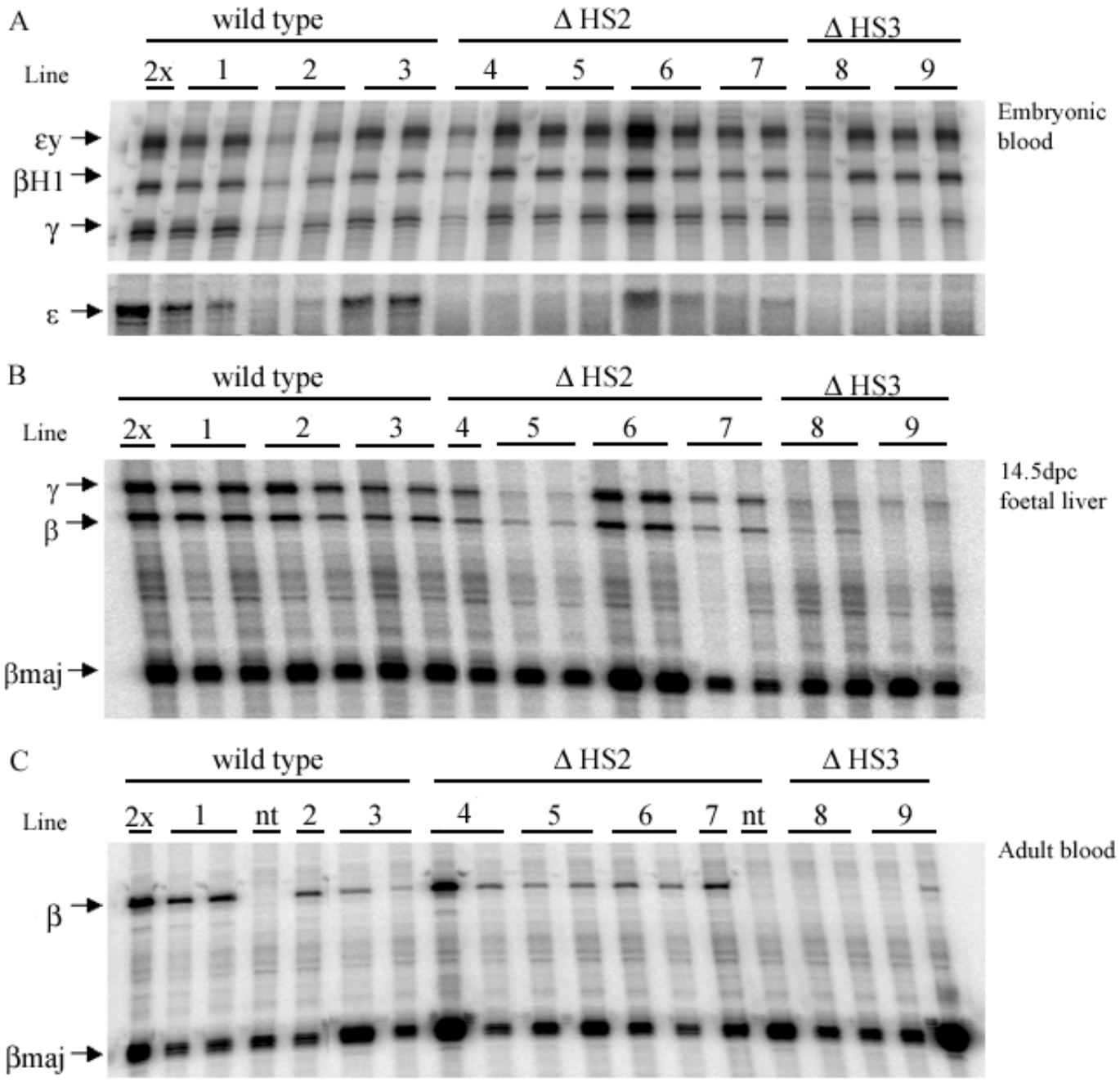

Figure 3*: S1 nuclease protection assays*—Panel A: S1 nuclease protection assay on RNA isolated from 10.5dpc yolk sacs of all single copy transgenic lines. Two littermates for each line are shown as well as a 2xRNA control from wild type line 1 for probe excess. The samples were assayed with radioactively labeled probes, for mouse εy and βH1 and for human ε and γ. The corresponding protected fragments are indicated with arrows. The lower panel shows a longer exposure of the human ε signal. The relative ratios of specific activities of εy:βh1:ε:γ probes were 5.2:1.9:1:2.7. Panels B and C: S1 nuclease protection assay on RNA isolated from 14.5 dpc foetal liver (B) and adult blood (C) of all single copy transgenic lines. Two littermates of each line were also tested, except for adult blood of lines 2 and 7. A 2x RNA control for probe excess is also shown. All samples were assayed with the same probe mix of radioactively labeled probes for mouse β_{major} and for human γ and β, with specific activitiy ratios of 3:5:1, respectively. Corresponding protected fragments are indicated with arrows. nt: non-transgenic controls.

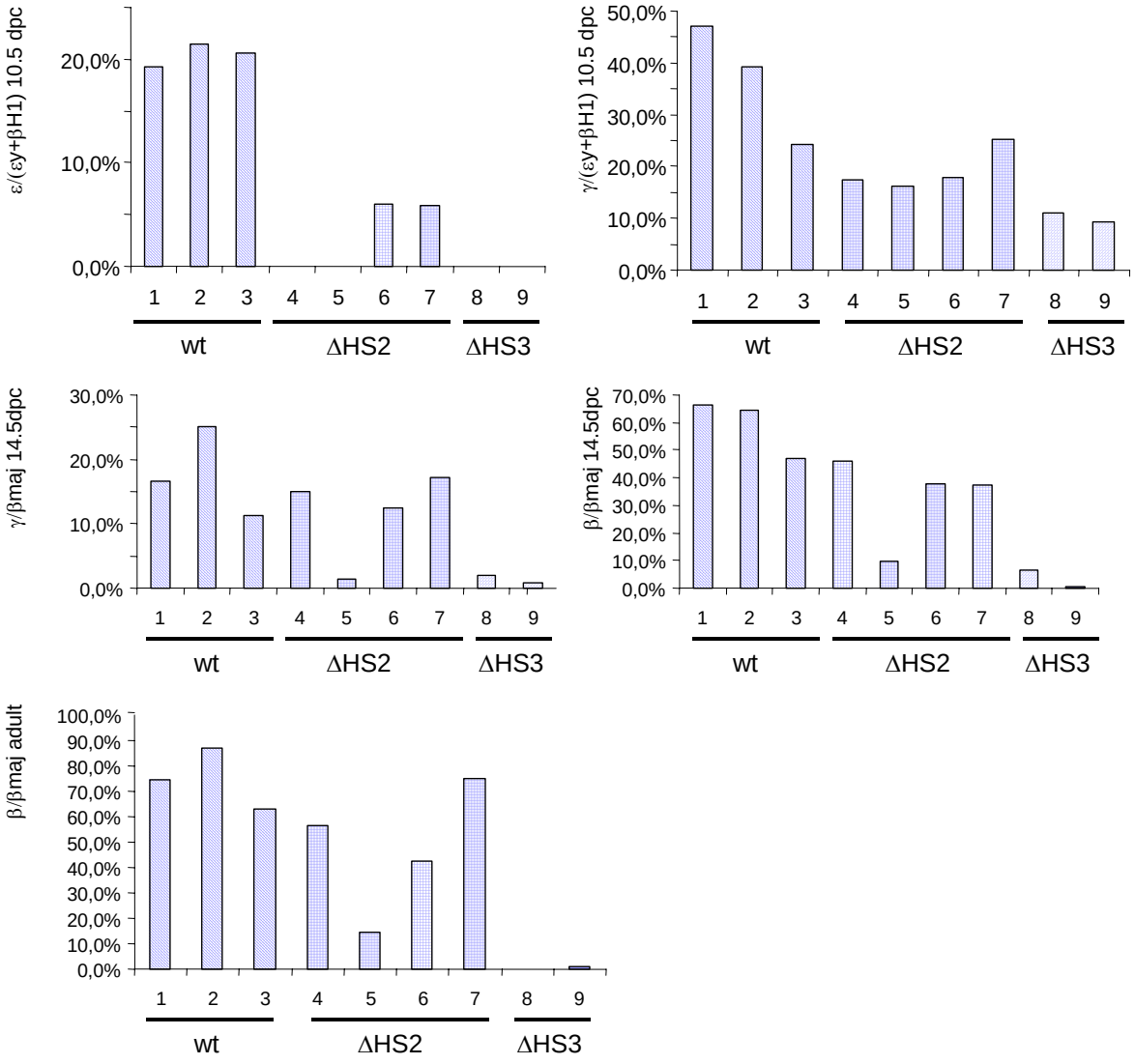

Figure 4*: Bar chart representation of the human globin expression levels at all developmental time points assayed as a percentage of the total mouse globin expression per transgenic line*—Line 1, 2 and 3 are wild type control lines, line 4, 5, 6 and 7 the HS2 deletion lines and line 8 and 9 the HS3 deletion lines.

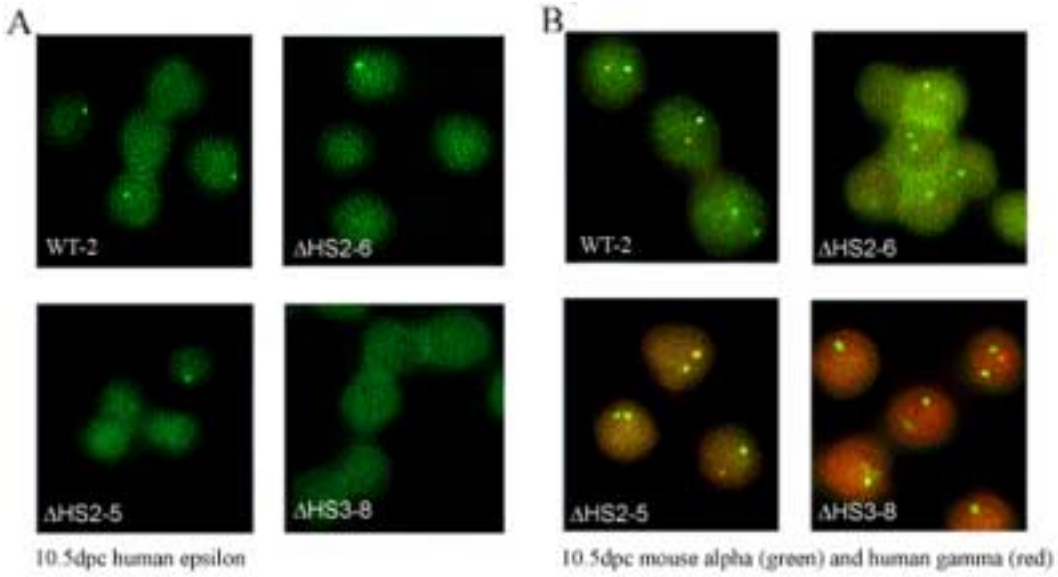

Figure 5*: In situ hybridisations on 10.5dpc embryonic blood*—A primary transcript *in situ* hybridisation using a human ε-globin intron specific probe (green signal) on wild type; ΔHS2 and ΔHS3. The deletion lines show less cells with a human ε signal. B primary transcript *in situ* hybridisation using a mouse α-globin intron specific probe (green signal) and a human γ-globin intron specific probe (red signal) on wild type; ΔHS2 (line 6); ΔHS2 (line 5) and ΔHS3. The wild type and ΔHS2-6 show comparable amounts of cells with human γ signal, ΔHS2-5 and ΔHS3-8 show clearly less cells positive for human γ.

Table 1*: Overview of transgenic mice*—In the table are listed; wild type line or deletion line, the initial copy number of the line, whether the line could be bred to single copy, the site of integration of the transgene and which line has been used in the analysis.

Trangenic line	copy nr	bred to single copy	integration site	used for analysis
wt β locus line 1	2	no	euchromatic	no
wt β locus line 2	3	no	telomeric	no
wt β locus line 3	3	no	euchromatic	no
wt β locus line 4	2	yes	centromeric	yes, line 1
wt β locus line 5	2	yes	subcentromeric	no
wt β locus line 6	1	_	subcentromeric	yes, line 2
wt β locus line 7	2	yes	telomeric	no
wt β locus line 8	2	yes	euchromatic	yes, line 3
ΔHS2 line 1	1	_	centromeric	yes, line 4
ΔHS2 line 2	4	yes	euchromatic	yes, line 5
ΔHS2 line 3	2	yes	telomeric	yes, line 6
ΔHS2 line 4	1	_	telomeric	yes, line 7
ΔHS3 line 1	3	no	subtelomeric	no
ΔHS3 line 2	2	no	centromeric	no
ΔHS3 line 3	2	yes	euchromatic	yes, line 8
ΔHS3 line 4	1	_	subcentromeric	yes, line 9

***Table 2**: primary transcript data 10.5dpc transgenic embryos*—The percentages of cells transcribing human ε-globin (n=200) and of cells transcribing both mouse α-and human γ-globin (n=200) are listed per transgenic line.

	primary transcript 10.5dpc	
transgenic line	cells expressing ε	cells expressing α and γ
wt β locus line1	17.5%	70%
wt β locus line2	13.5%	65.5%
wt β locus line3	16.6%	58.5%
ΔHS2 line 4	4.9%	66%
ΔHS2 line 5	6.1%	49.5%
ΔHS2 line 6	9.8%	62%
ΔHS2 line 7	9.7%	58%
ΔHS3 line 8	0%	36%
ΔHS3 line 9	0.8%	21%

***Table 3**: primary transcript data 14.5dpc transgenic embryos*—The percentages of cells transcribing both mouse β_{maj}-and human γ-globin (n=300) and of cells transcribing both mouse β_{maj}-and human β-globin (n=300) are listed per transgenic line.

	pimary transcript 14.5 dpc	
transgenic line	cells expressing γ and β_{maj}	cells expressing β and β_{maj}
wt β locus line1	16.6%	67.3%
wt β locus line2	25.1%	52%
wt β locus line3	11.3%	54%
ΔHS2 line 4	15%	49.5%
ΔHS2 line 5	1.4%	17.5%
ΔHS2 line 6	12.5%	49.3%
ΔHS2 line 7	17.2%	54.5%
ΔHS3 line 8	1.9%	3.5%
ΔHS3 line 9	0.9%	5%