Targeted Inactivation of the Major Positive Regulatory Element (HS-40) of the Human α-Globin Gene Locus

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We have examined the role of the major positive upstream regulatory element of the human α-globin gene locus (HS-40) in its natural chromosomal context. Using homologous recombination, HS-40 was replaced by a neo marker gene in a mouse erythroleukemia hybrid cell line containing a single copy of human chromosome 16. In clones from which HS-40 had been deleted, human α-globin gene expression was severely reduced, although basal levels of α1 and α2-globin mRNA expression representing less than 3% of the level in control cell lines were detected. Deletion of the neo marker gene, by using FLP recombinase/FLP recombinase target system, proved that the phenotype observed was not caused by the regulatory elements of this marker gene. In the targeted clones, deletion of HS-40 apparently does not affect long-range or local chromatin structure at the α promoters. Therefore, these results indicate that, in the experimental system used, HS-40 behaves as a strong inducible enhancer of human α-globin gene expression.

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GENESIS WITHIN THE human α- and β-globin clusters are coordinately expressed in erythroid cells throughout development. In each cluster, the structural genes are arranged along the chromosome in the order in which they are expressed during development. The α cluster (5'-ζ-α2-α1-b1-3') is located at the tip of chromosome 16p, and the β cluster (5'-ε-γ-Aγ-b-δ-3') is located on chromosome 11p.1,2 Rare, naturally occurring deletions3,4 first showed that α- and β-globin gene expression is dependent on regulatory elements associated with erythroid-specific DNase I hypersensitive sites located far upstream (5 to 40 kb) of the structural genes.5,6

In the β-globin cluster, there are four regulatory sequences (βHS1–4) collectively referred to as the β-globin locus control region (β-LCR). Each element binds tissue-restricted (GATA-1 and NF-E2) and, as yet unidentified, ubiquitous proteins.2,7,8 Constructs containing the β-globin gene linked to the β-LCR are regulated in a tissue- and developmental stage-specific manner. Furthermore, regardless of the position of integration in the mouse genome, the level of expression is copy number-dependent, each copy being expressed at a similar level to the endogenous mouse β-globin gene.9

In the α-globin cluster, there is a single regulatory element marked by an erythroid-specific DNase I hypersensitive site located 40 kb upstream of the embryonic ζ-globin gene, referred to as HS-40.10 This element lies in an intron of an anonymous, widely expressed gene located upstream of the α complex.10 HS-40 confers high levels of erythroid-specific expression of the α-globin genes in stable transfectants of mouse erythroleukemia (MEL) cells and in transgenic mice.

Detailed structural analysis has shown that HS-40 closely resembles HS2 in terms of the arrangement of sequence motifs for the binding of erythroid-restricted and ubiquitous-transacting factors.11-13 Nevertheless, important functional differences between HS-40 and the β-LCR have been reported in experiments using transfected MEL cells and transgenic mice. The level of expression of globin genes linked to HS-40 element is lower than that observed with the β-LCR, is not strictly dependent on the copy number of the integrated fragments, and tends to decrease during development.14,15 Therefore, it appears that HS-40 is not sufficient to produce full regulation of the α-globin gene cluster in transgenic mice, and it is possible that sequences other than HS-40 might be required. Such sequences could be associated with other erythroid-hypersensitive sites that have been mapped 4, 8, 10, and 33 kb upstream from the mRNA cap site of the ζ-globin gene.5 Previous studies have examined the role of these sites, which are attached to HS-40 and the α-globin genes in artificial constructs, but have failed to show modification in the expression of the linked genes.16 More recently, it has been shown that a 70-kb segment of DNA, spanning the entire α-globin cluster and containing all known erythroid-specific elements, does not allow full regulation of the α-globin cluster in transgenic mice.17

In previously studied human chromosome 16 × MEL hybrids, HS-40 was deleted by natural mutations that removed between 35 kb and more than 150 kb of DNA, including segments of the chromosome that have not been assayed in transgenic mice.11 It seemed possible that other elements that influence the structure and/or expression of the α cluster in vivo might exist in these regions. To test this hypothesis, a small segment of DNA containing HS-40 was deleted from the α cluster by homologous recombination. Thus, HS-40 was replaced by a neo marker gene in an MEL hybrid cell line containing one copy of human chromosome 16. This has enabled us to analyze more specifically the influence of HS-40 on α-globin expression, on the regulation of the widely expressed gene in which HS-40 is located, and on chromatin structure across the α-globin cluster. In addition, deletion of the neo marker gene, by using the yeast FLP recombinase system,19 proved that the α-globin gene expression in the targeted clones was not under the control of the selectable marker gene.

MATERIALS AND METHODS

Cell culture. Experiments were performed with the MEL hybrid cell line (LT 585P3), which contains a single copy of a normal
Fig 1. Homologous recombination into the human α-globin LCR. (A) Schematic representation of the human α-globin LCR showing the position of the HS-40 region and the arrangement of the α-globin coding genes on the chromosome 16. (B) Enlarged HS-40 region of (A) to show the targeted locus. (C) The targeting construct aligned with the chromosomal copy of this region. The neo gene is inserted between the two HpaI sites that flank the HS-40 element, and the HSV-tk (tk) gene is juxtaposed 3' of the isogenic homologous region (the isogenic homologous regions being represented in thick line). (D) Results of the homologous recombination process between genomic DNA (B) and the targeting vector (C). It should be noted that the insertion of the neo gene introduces a novel BamHI site into the mutated chromosome. Solid boxes represent the functional globin genes; hatched box represents the HS-40 hypersensitive site, and different probes used are indicated by dotted boxes. Restriction sites are indicated as follows: (B), BamHI; (Bg), BglII; (E), EcoRI; (Hp), HpaI; (H), HhaI; (PvuI).

Preparation of the targeting construct. The targeting vector is a 2.7-kb pUC 18-based plasmid in which an isogenic 7.9-kb sequence of human DNA was inserted. These human DNA fragments were obtained by screening an LT 585P3 genomic DNA library with a human DNA probe corresponding to HS-40. They include the 4-kb HindIII-HindIII fragment including the HS-40 site and the adjacent 5-kb HindIII-HindIII fragment located downstream of HS-40 (see Fig 1B). In the 4-kb HindIII-HindIII fragment, a 1.1-kb fragment including the HS-40 site was removed by the HpaI digestion and replaced by a 2.7-kb HindIII fragment that contained a neo marker gene. In this fragment, derived from pGEM-1-Fp-Neo plasmid, the neo gene is driven by a Friend retrovirus long terminal repeat (F-LTR) and is flanked on both sides by FLP recombinase target (FRT). In addition, a herpes simplex virus (HSV) thymidine kinase (tk) XhoI-HindIII cassette (HSV-tk), derived from pIC 19R/MCII-tk plasmid, was inserted at the 3' end of the 5-kb isogenic DNA fragment. Linearization at PvuI sites left sequences derived from the plasmid at both ends of the targeting construct.

Transfection of hybrid cells. One day before electroporation, cells were reseeded in fresh medium at a density of 10^6 cells/mL. For electroporation, cells were resuspended at 2 x 10^7 cells/mL in growth medium at 37°C. Cells were electroporated using 15 μg of the purified targeted vector to a final concentration of 5 nmol/L in cuvettes with 4-mm electrode gap using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, VA) adjusted to 500 microfarads and 300 V, giving an electric pulse of 800 V/cm for a time constant of 10.4 milliseconds. Immediately after electroporation, the cells were diluted into 10 mL of warm growth medium (37°C). Twenty-four
hours after electroporation, surviving cells were counted, pelleted, and plated in 96-well microtiter plates at 1,800 cells per well in 200 μL medium supplemented with G418 (700 μg/mL; Geneticin, GIBCO) and Gancyclovir (10 μg/mL; Syntex Research Co.). The number of G418-Gancyclovir-resistant clones in such wells was estimated, and cells were then distributed to obtain an average of 1 clone in every 2 wells. After selection and growth in microtiter plates using G418-Gancyclovir medium (14 to 20 days), individual clones were transferred into larger wells for expansion. When confluence was reached, half of the cells were removed and used for DNA analysis.

Identification of homologous recombinant clones. Homologous recombinants were identified by Southern blot analysis. DNA isolated from individual G418- and Gancyclovir-resistant clones was digested with BamH1. Approximately 10 μg of digested DNA was fractionated on a 0.8% agarose gel, transferred to nylon membrane (Hybond-N; Amersham, Arlington Heights, IL), and hybridized to the 32P-labeled probe A, a 0.8-kb Hpa I human DNA fragment corresponding to the sequence downstream of the HS-40 site (see Figs 1B, C, and D).

To confirm the results of the first Southern blot analysis, DNA isolated from potential homologous recombinant clones was submitted to BamH1 or Bgl II digestion. After migration on agarose gel and transfer to nylon membrane, the digested DNA was hybridized to either the 32P-labeled probe B, probe C, or probe D. Probe B is a 1.1-kb Hpa I fragment complementary to HS-40 (see Fig 1B); probe C is a 1.5-kb Pst I human α-globin gene fragment that hybridizes simultaneously to α1- and α2-globin genes (see Fig 1A); and probe D is an 850-bp neo gene-coding fragment (see Fig 1D).

Isolation of clones with an FLP-mediated deletion of the selectable marker. In one of the homologous recombinant clones, deletion of the F-1LTRneo gene was obtained after transient FLP recombinase expression. This was initiated by electroporation of the homologous recombinant clone with the pCFIZ construct that coexpresses FLP and β-galactosidase from the same mRNA.19 Forty-eight hours after electroporation of 40 μg of pCFIZ construct, the cells were assayed and sorted for β-galactosidase expression by "FACS-Gal" assay. The clones sensitive to G418 were analyzed by Southern blot for FLP-mediated deletion of F-1LTRneo. This analysis was performed with probe D as previously described and probe E (1.4 kb EcoRI-HindIII fragment shown in Fig 5A).

Analysis of RNA. Total cellular RNA from uninduced and HMBA-induced cells was prepared by the method of Chomczynski and Sacchi,20 and, for Northern blots, poly(A) mRNA was subsequently selected using the polyAT tract mRNA isolation system (Promega, Madison, WI). The amount of human and mouse α-globin mRNA was then determined by quantitative RNase protection assays21 using the following labeled antisense RNA probes: (1) a human αc' riboprobe that includes 231 nucleotides (nt) complementary to the 5' end of the α1 and α2 mRNA from nucleotide -66 upstream of the mRNA cap site to nt +165 in the first intron of α1- and α2-globin genes. This probe is expected to produce two similar protected fragments of 133 nt with α1 and α2 mRNA; (2) a human α2-specific riboprobe that includes 290 nt complementary to the 3' end of the α2 mRNA, starting from the single HindIII site at the end of exon 2 until the poly(A) sequence. Because of the divergence in the 3' untranslated region between α1 and α2 mRNA, this probe is expected to produce two different protected fragments, a 270-nt fragment specific to the α2 mRNA and a shorter 170-nt fragment specific to the α1 mRNA; (3) a human η1-globin riboprobe that includes 133 nt complementary to the 3' end of the exon 3. This riboprobe, starting from the BstE11 site and extending to the poly(A) site, is entirely protected by the η1 mRNA; (4) a mouse ma-globin riboprobe that includes the entire exon 1 of the mouse α-globin gene and that is expected to give a protected fragment of 190 nt after hybridization with mouse α-globin mRNA; and (5) A mouse mar'-globin riboprobe that includes 180 nt complementary to the 3' end of the first exon of the mouse α mRNA. This probe is expected to produce a protected fragment of 75 nt.

Specific protected fragments were quantitated by densitometric analysis of autoradiographs using a Bioprofil 4.6 densitometer (Vilber Lourmat) standardized against mouse α endogenous RNA. Each quantitation was performed in duplicate on different preparations of mRNA and using different exposures of the autoradiograph. For Northern blots, approximately 2 μg of poly(A)-selected RNA was analyzed after electrophoresis through 1.0% agarose denaturing formaldehyde gels. RNA transfer was achieved by blotting in 10× SSC (1× SSC, 0.15 mol/L NaCl and 0.015 mol/L Na citrate) to Hybond-N (Amersham) as described in Sambrook et al.22 Hybridization was performed in a buffer containing 5% formamide, 1× SSC, 1× Denhardt’s, 7.4% dextran sulfate, and 20 μg/mL salmon sperm DNA at 42°C overnight, using either an Sph 1-HindIII cDNA fragment corresponding to exons 5-7 from the ubiquitously expressed gene at coordinate -14 of the α-cluster or a cDNA probe (1A1) corresponding to human tuberin.23 Filters were washed in 0.1× SSC and 0.1% sodium dodecyl sulfate at 45°C for 5 minutes for the -14 probe or at 65°C for 10 minutes for the tuberin probe.

Analysis of DNAse I hypersensitive sites. To study the pattern of DNAse I sensitivity, nuclei were prepared from approximately 106 MEL cells, normal (LT S85P3) or abnormal (HR1) hybrid cells, and analyzed as set out in Higgs et al.13 and modified in Craddock et al.24 The probes used include the α-globin 3' hypervariable region (3'HVR), α-globin, L1, L4, RA1.4, 5'HVR, HTF0.9, RA2.2, and Bgl6.25,26 The φα1 probe was prepared by PCR amplification from the plasmid pH6 (a Pst I fragment containing the φα1 gene in pBR322) as described by Craddock et al.24

RESULTS

Isolation of homologous recombinants. By double crossover, a targeting vector was designed to replace the 1.1-kb Hpa I fragment containing HS-40 with a neo marker gene,
Fig 3. Verification of the homologous recombinant clones by different Southern blots. Targeting clones (HR1 and HR2), nonhomologous recombinant (NHR), parental hybrid cells (LT), and negative control (MEL), were digested by BamHI or Bgl II enzymes and then hybridized with different probes. (A) Hybridization with the HS-40 probe B (Fig 1B) is used to ascertain that the HS-40 element is missing in clones HR1 and HR2. Probe B detects a 19-kb band and an 8.8-kb band in parental DNA (LT) and in nonhomologous recombinant DNA after digestion by BamHI and Bgl II, respectively. (B) Probe C (Fig 1A) is used to verify the presence of the chromosome 16 and detects a 14-kb band after digestion by BamHI and detects two bands (12.5 kb and 7.5 kb) after digestion by Bgl II. (C) Probe D (Fig 1D), corresponding to the neo gene, is used to confirm the presence of this marker gene in place of the HS-40 region in clones HR1 and HR2. It detects a 9.4-kb band after digestion by BamHI and a 10.2-kb band after digestion by Bgl II, in homologous recombinant DNA.

The positive-negative selection strategy described by Mansour et al. was used to isolate homologous recombinants.

The replacement-type targeting construct was introduced by electroporation into a human/MEL hybrid cell line containing one copy of chromosome 16 (LT 585P3). Selection of 3 x 10^6 cells surviving electroporation with G418 and Gancyclovir yielded 360 clones resistant to both drugs (G418R and Ganc'). Each clone was amplified separately and analyzed by Southern blot after digestion with BamHI and hybridization with probe A, which is located inside the vector (Fig 1). When a single integration of the vector occurs at a random site, probe A should show two fragments, a 19-kb genomic fragment that corresponds to the endogenous sequence derived from human chromosome 16 and a fragment of 7 kb corresponding to the vector inserted randomly into the genome. When a targeting event occurs, probe A should show a unique BamHI fragment of 10.7 kb in place of the normal 19-kb fragment because of the presence of a BamHI site within the neo gene (Fig 1).

Among the 360 G418R Ganc' clones analyzed, two (HR1 and HR2) showed the expected pattern for correctly targeted clones (Fig 2). To verify that the HS-40 element had been deleted in HR1 and HR2, DNA was digested by BamHI or Bgl II and hybridized with probe B (Fig 1B). Fragments of the expected size were detected in the parental cell line LT 585P3 (LT) and in a nonhomologous recombinant clone (NHR) but not in HR1 and HR2 (Fig 3A). To show that this was not simply because of the loss of human chromosome 16, the same membrane was rehybridized with probe C, specific to the human a-globin gene (Fig 1A). Fragments of the expected size observed after BamHI and Bgl II digestions proved the presence of chromosome 16 in all of the hybrid clones (Fig 3B). Using probe D, we confirmed that the clones HR1 and HR2 contain the insertion of the neo gene in place of the HS-40 region (Fig 3C).
**α-Globin gene expression in homologous recombinant clones.** Total cellular mRNA was prepared from the two targeted clones HR1 and HR2, from the parental LT 585P3 cell line (LT), and from a nonhomologous recombinant clone (NHR), before and after induction of differentiation of the cells by HMBA. Globin mRNA content was determined by RNase protection assays.

To estimate the level of human α-globin gene expression after induction, we used the mouse α-globin mRNA level as an internal standard. Mouse and human α-globin mRNA were assayed simultaneously with two probes of the same specific activity using the same preparations of mRNA. The ratios of human α/mouse α-globin mRNA found in the hybrid cells were then corrected, taking into account the diploid complement of mouse chromosomes versus a single human chromosome 16.

We did not detect any significant difference in the levels of expression of human α1- and α2-globin mRNA between targeted (HR1 and HR2), parental (LT), and nontargeted (NHR) control cells before induction (Fig 4A). After induction, the level of human α mRNA in the normal hybrid cell line was approximately equal to that of the endogenous
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Fig 5. Excision of the neo marker gene. (A) Schematic representation of the human α-globin locus showing the expected bands, after double EcoRI-Bgl II digestion of the genomic DNA, in parental hybrid cells (LT), in homologous recombinant clone (HR1), and in the HR clone deleted of the neo gene (C10). (B) Southern blot analysis of genomic DNA. Putative clone deleted of the neo marker gene (C10), targeted clone (HR1), parental hybrid cells (LT), and negative control (MEL) were digested by EcoRI and Bgl II enzymes and then hybridized with different probes. Probe E detects a 4.8-kb band in HR1 replaced by a 2-kb band in C10; probe D is used to verify the absence of the neo gene in the C10 clone.

Accurate quantification of α2-globin gene expression was compromised in this RNase protection assay, because the signal was divided into two fragments. Therefore, we used a second human riboprobe ‘αc’ that protects both α2 and α1 mRNA, producing fragments of identical sizes (data not shown). Using this assay we also estimated α-globin mRNA expression in the HR clones to be only 3% of the level of mRNA in control cell lines after induction of erythroid differentiation.

The level of human θ1-globin mRNA in the presence or absence of the HS-40 region was determined by quantitative RNase protection assay using the specific human θ1 riboprobe (Fig 4B). As for α1- and α2-globin gene expression, we were unable to detect any difference in the expression of the θ1-globin gene in the normal and mutant hybrids before induction. θ1-mRNA expression did not increase sig-
Expression of the ubiquitous gene containing HS-40. The HS-40 element is located within an intron of a gene located 14 kb upstream of the \( \zeta \)-globin mRNA cap site. We examined expression of this gene by Northern blot analysis to determine if it was affected by the HS-40 knockout. Approximately equal amounts of poly(A)-selected mRNA were loaded onto each track of the Northern blot; the relative

Fig 6. RNase protection analysis of \( \alpha \)-globin gene expression in the targeted clones with (HR1) or without (C10) the neo gene and in control cell lines (LT and MEL). RNA were hybridized with human \( \alpha \)2-globin RNA probe (ha1) and mouse \( \alpha \)-globin RNA probe (ma'). The location and size of the protected fragments are indicated. (The faint difference in size observed for the band in the second lane is because of the presence of ethanol traces in the sample). (+), presence of HMBA; (−), absence of HMBA.

Fig 7. (A) and (B) show Northern blot of poly(A)-selected mRNA from MEL cells, the normal chromosome 16 × MEL hybrid (LT) and abnormal hybrid (HR1). The probes used are shown below. (C) The agarose gel stained with ethidium bromide before Northern blotting is shown.

Isolation and analysis of an HR clone after deletion of the selectable marker. In clone HR1, we used the FLP-recombinase to catalyze recombination between the FLP recombinase target (FRT) sequences and, consequently, deletion of the neo marker gene. Among the 58 viable clones obtained after fluorescence-activated cell sorting of the electroporated cells, only one was G418\(^{\text{R}}\) (clone C10). By Southern blotting, we verified that, in C10 clone, the F-LTR/neo gene was indeed deleted (Fig 5B). As expected, we observed with probe E a conversion of the 4.8-kb band in HR1 into a 2-kb band in C10; and with probe D, which is specific of the neo gene, we noted the absence of band.

To compare the level of human \( \alpha \)-globin gene expression in homologous recombinant clones with (HR1) or without (C10) the neo marker gene, we used the \( \alpha \)2 probe in RNase protection experiments. We did not detect any difference in the level of expression of human \( \alpha \)-globin mRNA between HR1 and C10 clones (Fig 6). As in HR1, before and after induction, a very low level of \( \alpha \)-globin mRNA was observed in the HR clone without the neo gene.
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A

LT

HR1

Bam HI
Probe L4

LT

HR1

Bam HI
Probe ω2

Fig 8. Analysis of DNase I hypersensitive sites in the normal (LT) and targeted (HR1) hybrid cells. The digest and probe used are indicated below. (A) Analysis of DNase I hypersensitive sites located at -40, -35, and -33 in uninduced hybrids. Because a novel BamHI site is contained within the inserted neo gene in HR1, the BamHI fragment in this mutated chromosome DNA is shorter than normal. (B) Analysis of DNase I hypersensitive sites located at +22, +20 and +17 in both uninduced (U) and induced (I) hybrids.

loading was estimated by ethidium bromide staining and by hybridization to the human tuberin probe (Fig 7). After hybridization to an Sph I-HindIII cDNA fragment corresponding to exons 5-7 of the -14 gene, two previously described mRNA transcripts (2.5 and 3.2 kb) were detected. In the normal hybrid (LT 585P3) the 3.2-kb transcript was predominant. In the MEL control, faint bands were observed, presumably representing the mouse homologue of this gene. In the targeted hybrid (HR1), two bands of diminished intensity, but more intense than MEL alone, were observed. Induction of the MEL hybrids appeared not to affect the level of the 2.5- or 3.2-kb transcripts when compared with the corresponding tuberin (1A1) signal.

Analysis of DNase I hypersensitive sites. We analyzed previously described constitutive and erythroid-specific DNase I hypersensitive sites throughout a region of approximately 200 kb including the α-globin cluster (Figs 8A and B and Fig 9). We analyzed both uninduced and induced hybrids corresponding to the normal and targeted chromosome. All sites analyzed, including those at the α-globin promoters (Fig 8B), were present and of equal intensity in both hybrids (Figs 8 and 9).

DISCUSSION

It has been reported previously that large deletions removing a region upstream of the human α-globin gene cluster are associated with severe downregulation of both α genes. All such deletions remove a region of approximately 20 kb, extending from approximately 33 kb to approximately 52 kb upstream of the ζ mRNA cap site.28 These findings support the notion that positive regulatory sequences exist in this segment of DNA. It has been previously shown, using a hybrid MEL cell line [(αα)8] containing a copy of human chromosome 16 from which there is a large natural deletion of 62 kb (from coordinates -52 kb to +10 kb), that expression of the human α-globin genes is reduced to a low but detectable level, representing less than 1% of the mouse α-globin mRNA after induction of the cells.3

Until now, natural deletions provided the only model to examine the effect of deleting the major α-globin control element in its chromosomal context. Although all of the natural mutations delete HS-40, they also remove extensive segments of DNA including other erythroid-specific DNase I hypersensitive sites normally located 33 kb, 10 kb, 8 kb, and 4 kb upstream of the α-globin cluster, causing a major perturbation of the normal structure of the α complex. To evaluate the role of the HS-40 element in its natural chromosomal context with as little disruption as possible, we decided to remove by homologous recombination a small, 1.1-kb fragment containing HS-40. We achieved this by mutating a human/MEL hybrid cell line containing a normal copy of chromosome 16, using the positive-negative selection homologous recombination strategy,27 and by introducing a neo marker gene in place of the HS-40 region. Using this strategy, we obtained two correctly targeted clones.

Comparison of human α-globin mRNA expression in normal LT and targeted clones showed that the level of human α-globin mRNA expression is severely reduced when HS-40 is deleted. However, low levels of α-globin mRNA were detectable before and after induction of the targeted cells that represent approximately 3% of α-globin mRNA expression in normal cells (Fig 4A). To test whether these low levels of α-globin gene expression were because of the neo gene left in place of the HS-40 element, the selectable marker
Fig 9. Summary of DNase I hypersensitive sites analyzed in the hybrids LT 56SP3 (LT) and in the targeted clone (HR1). Above, the telomeric region of chromosome 16p is shown with the telomere (a) and the subtelomeric region (I). Globin genes, (II): α1 gene, (III): anonymous widely expressed genes, (C); MPG gene. The direction of transcription of each gene is shown by arrows. HS-40, (I). The scale is in kilobases.

The locations of previously reported erythroid-specific and constitutive hypersensitive sites are shown by arrows. The positions of probes used in this study are shown below, and, at the bottom of the diagram, the plus sign (+) indicates the presence of hypersensitive sites in the hybrids studied. The box marked neo (not to scale) indicates that HS-40 was replaced by the neo gene in this hybrid.

1. was removed by using FLP recombinase/FRT system in one of the two homologous recombinant clones. Comparison of the α-globin mRNA expression with the clone that still contain the neo gene led us to conclude that the severe reduction of α-globin gene expression and the basal expression observed are not under the influence of the selectable marker but are caused by the removal of HS-40. Expression of the previously described fetal/adult θ-globin gene is also considerably reduced in the targeted clones HR1 and HR2. This result provides the first evidence that expression of the θ1 gene is under the influence of the HS-40 region, as are the other genes of the human α-globin cluster. Together, these findings extend previous observations on the naturally occurring mutants and show that there are no other sequences outside of the HS-40 element that, on their own, are capable of exerting a major enhancer effect on expression of the α genes. However, these findings do not exclude the possibility that other sequences may synergize the effect of HS-40.

The observation that low, basal levels of human α-mRNA expression are seen in the absence of HS-40 both in these experiments and in the (αβ)AA mutant is interesting and consistent with the observation that the hypersensitive sites located at the α-globin promoters are present and of equal intensity in the normal and targeted hybrids. These findings confirm that HS-40 is not required for these hypersensitive sites to form in vivo and imply that HS-40 does not exert its effect by remodelling the local chromatin structure at the α promoters. Similarly, we found that other erythroid-specific sites (at −33) still form in the absence of HS-40.

HS-40 lies within an intron of a widely expressed gene, and, therefore, it was of interest to examine the effect of HS-40 on expression of this gene. Unlike the α genes, in the HR1 clone, this gene continued to be expressed in the absence of HS-40, albeit at a reduced level. Preliminary results indicate that the expression of this ubiquitous gene is identical in the parental cell line (LT) and in the homologous recombinant clone without the neo marker (C10). Thus, it seems that the reduction in the level of expression in HR1 is because of the disruption of the widely expressed gene caused by placing an actively transcribed neo gene in its intron, rather than because of the absence of the HS-40 element. Hence, the effect of the HS-40 disruption seems to be essentially local and not caused by a global loss of chromosome 16 transcription in the targeted cells. It is also interesting to note that the constitutive DNase I hypersensitive site associated with the promoter of this gene (at coordinate −14) formed normally, as did all constitutive sites examined, confirming that deletion of HS-40 appears to have no effect on long-range chromatin structure.

These results suggest that the 1.1-kb region including HS-40 behaves in its natural chromosomal context as a strong inducible enhancer of human α-globin gene expression and, unlike the β-LCR, does not appear to influence long-range or local chromatin structure.

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