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Localisation of *cis* Regulatory Elements at the β-Globin Locus: Analysis of Hybrid Haplotype Chromosomes

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Several *cis* elements at the β-globin gene cluster and the upstream locus control region (LCR) have been implicated in modulation of fetal haemoglobin (Hb F) level in β-globin disorders. To determine the role of elements at the LCR and the β-globin gene cluster on HbF level among sickle cell anaemia (SCA) patients, hybrid hapotype β^S chromosomes exhibiting variation in the association of alleles of LCR hypersensitive site 2 (HS2) and the β-globin gene cluster restriction fragment length polymorphosim (RFLP) haplotypes were identified in an unselected population of 100 patients. On 15 chromosomes the polymorphic HS2 short tandem repeat $(TA)_xN_{10-12}(TA)_y$ containing a Hox2 binding motif differed from that typically associated with the corresponding β-globin gene cluster RFLP haplotype. Among patients homozygous for the Benin RFLP haplotype, in whom one chromosome carried the $(TA)_9N_{10}(TA)_{10}$ allele, no effect on HbF level was observed. Polymorphism of the pre-$^G\gamma$ framework, an enhancer located 25 kb downstream of HS2 localised the breakpoint for each of these 'hybrid' haplotype chromosomes upstream of this element. Previously described hybrid haplotype chromosomes with the $(TA)_9N_{10}(TA)_{10}$ HS2 allele associated with raised HbF by contrast arise by recombination 1 kb downstream of the pre-$^G\gamma$ framework. This study suggests that variability in HbF level associated with polymorphisn of the HS2 enhancer depend on downstream determinant (s) in tight linkage disequilibrium with HS2. The pre-$^G\gamma$ framework is the only known polymorphic *cis*-active determinant in this region. © 1999 Academic Press

The level of fetal haemoglobin (HbF; $\alpha_2\gamma_2$) differs widely among patients with sickle cell anaemia (SCA). This variation has significance on the haematological and clinical course of the diseases (1, 2). Higher HbF levels are correlated with improved clinical outcome (3, 4) providing a rationale for therapeutic induction of HbF (5, 6).

Cis-active (7-10) and *trans*-acting (11-14) genetic determinants have been implicated in modulation of Hb F level in SCA. Among the major sickle cell anaemia haplotypes, defined by restriction fragment length polymorphism (RFLP) in the β-globin gene cluster (15-18) HbF is lower in patients with Benin, Central African Republic (CAR) or Cameroon RFLP haplotypes (less than 10%) than in those with the Senegal or Asian/Saudi-Arabian haplotypes (15-30%). This difference may reflect polymorphic variation in regulatory *cis*-elements which alter the binding of proteins that activate or repress transcription thereby shifting the reciprocal balance between β^S- and γ-globin gene expression. In support of this, haplotype-linked polymorphic variants of a tandem repeat sequence $(AT)_X(T)_Y$ located −530 bp from the β-globin gene cap site are bound by the silencer protein BP1 with different affinities (19). The binding affinity of BP1 is highest for the Asian and lowest for the CAR alleles which *in vivo* are associated with high and low levels of HbF respectively in β^S homozygotes (20).

Inter-haplotypic variation of *cis* elements which directly modulate γ-globin gene transcription is less well defined. A C → T polymorphism at −158 bp from the $^G\gamma$-globin gene creates a *Xmn* I restriction site which distinguishes 'high HbF' haplotypes including Senegal and Asian β^S chromosomes. No regulatory function, however, has been ascribed to this polymorphism (21). Haplotype specific polymorphisms in the 3′ $^A\gamma$-globin gene enhancer, 5′ flanking sequences (−1227 to +53 bp of $^G\gamma$ and −1279 to +53 bp of $^A\gamma$) and intron 2 of both γ-globin genes do not appear to influence HbF level in SCA (22-24). A putative regulatory element which contains binding motifs for both ubiquitous and erythroid-specific transcription factors located −1450 to −1225 bp from the $^G\gamma$-globin gene cap site (pre-$^G\gamma$ framework) has been described (Fig. 1). Haplotypic variation

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within GATA-1 and cyclic adenosine monophosphate (cAMP) response element (CRE) consensus sequences of the pre-$^{G}\gamma$ framework results in differential binding affinity of the cognate proteins *in vitro* which is correlated to HbF level (25).

Sequence variation within the β-globin locus control region (LCR), located between 6 and 20 kb 5′ to the cap site of the ϵ-globin gene (26, 27) has been postulated to alter γ-globin gene expression. Each of four erythroid specific DNase I hypersensitive sites (HS-1 to 4), which define the LCR, contains multiple *cis*-active elements (28-31). Polymorphism of multiple *cis*-active elements including the binding sites for Hox2 and Sp1 transcription factors (Fig. 1) which are in linkage disequilibrium with individual sickle haplotypes has been implicated in the variability of HbF level in SCA (32-35), β-thalassaemia (36), hereditary persistence of HbF (37, 38) and in the number of HbF containing cells (F-cells) in Hb AS heterozygotes (39). In SCA this conclusion has been drawn from observations that variant β^{S} chromosomes with Senegal HS2 and downstream Benin elements are associated with HbF levels typical of the Senegal haplotype (32, 34). Evidence that HS2 polymorphism directly modulates Hb F level or γ-globin gene transcription, however, is lacking.

To elucidate the effect of variation within *cis* elements on HbF level hybrid haplotype β^{S} chromosomes in which the association between alleles at HS2 and the β-globin gene cluster is discordant were characterised. The hybrid haplotype β^{S} chromosomes identified provide an opportunity to dissect the contribution of individual functional *cis* elements in the LCR and the β-globin gene cluster to modulation of HbF level in SCA.

RESEARCH DESIGN AND METHODS

Patients and samples. Blood was obtained with informed consent from one 100 patients (51 male, 49 female) with SCA aged 1-18 years including eight sib-pairs. Eighty-nine patients originated from West Africa and 11 from the Caribbean islands. HbF level was measured by automated high performance liquid chromatography using a six minute gradient program (Bio-Rad Variant, Hercules, CA). DNA was extracted from blood by a standard procedure.

Length variation of HS2 polymorphism. The LCR HS2 short tandem repeat (STR) polymorphism $TA_xN_{10-12}TA_y$ (Fig. 1) containing the binding site for Hox2 was PCR amplified from 1.0 μg of genomic DNA using 200 ng each of primer H1 and H2 (H1: 5′-GATGCCTGAGACAGAATGTG-3′; H2; 5′-TATAGAGGCCACCTGCAAG-3′) and 20 ng ^{32}P-labelled H1 primer. Primer H1 (500 ng) was 5′-end labelled with γ^{32}P-ATP (3000 Ci/mmol; Amersham, UK) using T4 polynucleotide kinase (Promega, Southampton, UK); unincoporated isotope was removed by gel filtration (Sephadex G-50; Pharmacia Biotech, St. Albans, UK). PCR conditions were initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 92°C for 30 s, annealing at 64°C for 45 s, extension at 72°C for 90 s and a final extension at 72°C for 3 min. PCR product (20μl) was dried and reconstituted with 10 μl loading buffer (80% formamide containing 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol, 20 mM sodium hydroxide and 1 mM EDTA) and 4 μl applied to an 8% denaturing polyacrylamide gel (Promega). Following electrophoresis the gel was dried at 80°C under vacuum and exposed to X-ray film (Kodak BioMax MR, Cambridge, UK) at room temperature overnight.

Single strand conformation polymorphism analysis of HS2 STR and pre-$^{G}\gamma$ framework polymorphism. Single strand conformation polymorphism (SSCP) analysis was used to distinguish HS2 STRs linked to CAR and Senegal haplotypes which are identical in length (48 bp). PCR amplification was performed as described above incorporating α^{33}P-dATP (8 Ci/mmol; Du Pont, Stevenage, UK). A region flanking three polymorphic sites, nucleotides −1450, −1280 and −1225 in the pre-$^{G}\gamma$ framework (Fig. 1), was PCR amplified as a single DNA fragment from genomic DNA (1.0 μg) using 200 ng each of primer B1 and B2 (B1: 5′-ACTGGAAGATACTGATAATTGGAC-3′; B2: 5′-CTCGATCCATGACCTTGG-3′) incorporating α^{33}P-dATP (8 Ci/mmol, Du Pont). PCR conditions were initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 92°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 90 s and a final extension at 72°C for 3 min. PCR product mixed with an equal volume of loading buffer was heated to 92°C for 2 min and chilled on ice for 5 min. Samples (5 μl) were electrophoresed on a native polyacrylamide gel (4.5%) at constant temperature (4°C) and power (40W).

DNA sequencing. Double-stranded DNA sequencing was performed on PCR amplified DNA. To characterise individual HS2 STR alleles in subjects heterozygous for the polymorphism PCR amplified product was cloned into a T-vector using a commercially available kit (Invitrogen, San Diego, CA). DNA clones were distinguished according to insert size by digesting with *Eco*R I. Dideoxy DNA cycle sequencing (*fmol,* Promega) was performed using 20 ng of a 5′-end ^{32}P-labelled amplification primer and the cycling conditions described above. Sample electrophoresis was on an 8% denaturing polyacrylamide gel.

RFLP haplotype analysis. The β-globin gene cluster RFLP haplotype was determined by restriction enzyme analysis of PCR products flanking the following polymorphic sites: *Hinc* II 5′ to the ϵ gene, *Xmn* I 5′ and *Hin*d III in the $^{G}\gamma$ gene, *Hin*d III in the $^{A}\gamma$ gene, *Hin*c II in and 3′ to the $\iota\beta$ gene, *Ava* II in and *Hin*f I and *Hpa* I 3′ to the β. Structural analysis of α-globin genes was performed by Southern blot hybridisation of *Bgl* II and *Bam* HI digested genomic DNA with ζ and α-globin probes respectively.

RESULTS

One hundred and ninety-five of the 200 chromosomes studied conformed to one of the classical β^{S} RFLP haplotypes. Of these 15 chromosomes (7.7%) showed loss of the expected haplotypic association with the corresponding HS2 STR allele. A single band corresponding to the 42 bp HS2 STR allele characteristic of the Benin haplotype (Fig. 2, lane 1) was observed in 158/172 (91.9%) chromosomes which displayed a Benin RFLP pattern. A patient homozygous for the CAR RFLP haplotype was found to have a Benin HS2 allele on one chromosome (lane 2). The expected 50 bp Cameroon HS2 STR allele (33) was present but the Benin allele absent, in two siblings heterozygous for the Benin/Cameroon RFLP haplotype (lanes 3 and 4). In a subject compound heterozygous for Benin and Senegal RFLP haplotypes the Benin allele was absent and a 48 bp HS2 STR allele was visible (lane 11). Eleven other patients homozygous for the Benin RFLP haplotype were found to have a second band distinct from the Benin allele. This consisted of a 48 bp allele in seven

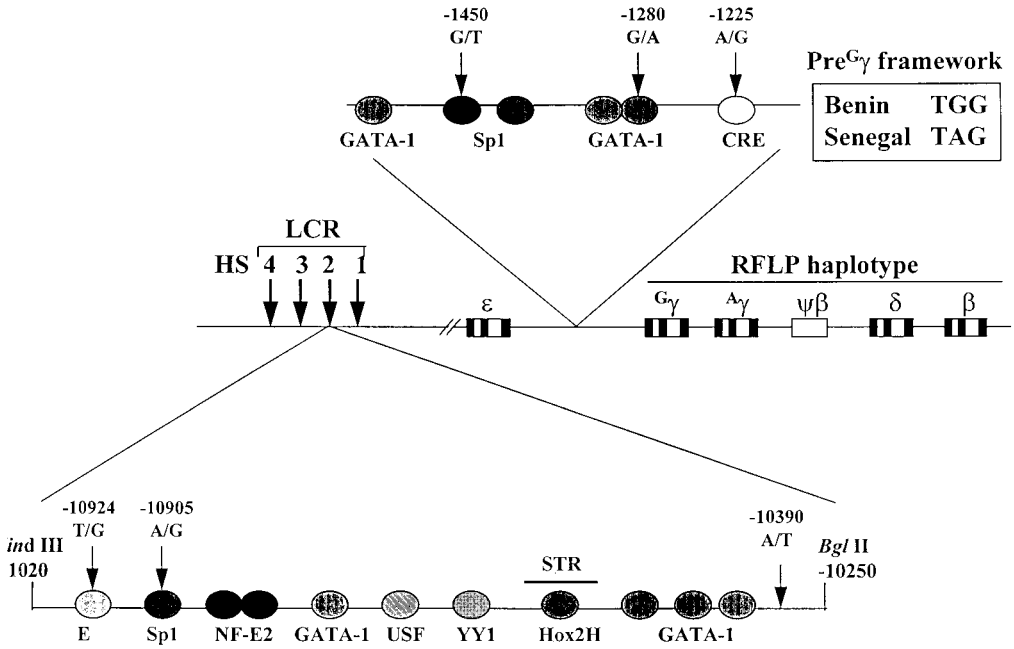

FIG. 1. Schematic diagram showing the location of polymorphic *cis*-acting elements in the locus control region (LCR) hypersensitive site 2 (HS2) and the pre$^{G}\gamma$ framework relative to the interval defined by RFLP haplotypes in the β-globin locus. Polymorphic nucleotides in HS2 and the pre$^{G}\gamma$ framework are numbered relative to the cap sites of ϵ- and $^{G}\gamma$-globin genes respectively. The solid bar and STR denotes the region of HS2 containing the repeat motif $(TA)_xN_{10-12}(TA)_{12}$ in linkage disequlibrium with common RFLP haplotypes. The three nucleotides constituting the pre$^{G}\gamma$ framework polymorphism, shown boxed in the order −1450, −1280 and −1225 correspond to the unique RFLP haplotypes specified.

cases (lanes 5-9, 12 and 14) a 50 bp allele in three (lanes 10, 15 and 16) and a 52 bp allele in one case (lane 13). The distribution of β^{S} chromosomes is summarised in Table 1.

Sequencing confirmed the configuration of nucleotide repeats to be the same as previously reported for the HS2 STR alleles characteristic of Benin $(TA)_8(CA)_2(TA)_2CGTG(TA)_7$ and Senegal $(TA)_9(CA)_2(TA)_2CG(TA)_{10}$ (Fig. 3) (32, 33). The sequence of the Benin and Senegal alleles shown is that of cloned PCR product from patient F known to be homozygous for the Benin RFLP haplotype. The DNA sequence of the CAR HS2 STR $(TA)_8(CA)_2(TA)_2CG(TA)_{11}$ characterised concurs with one report (40) but differs from another in which an additional 5′ TA dinucleotide was described (33). Heterogeneity at the 5′ end of the motif linked to the CAR haplotype shows further diversity among CAR chromosomes. Since the Senegal and CAR STR are identical in length (48 bp) but differ in sequence these alleles were distinguished by PCR-SSCP analysis (Fig. 4; upper panel). Thirteen chromosomes with a CAR HS2 STR allele were as-

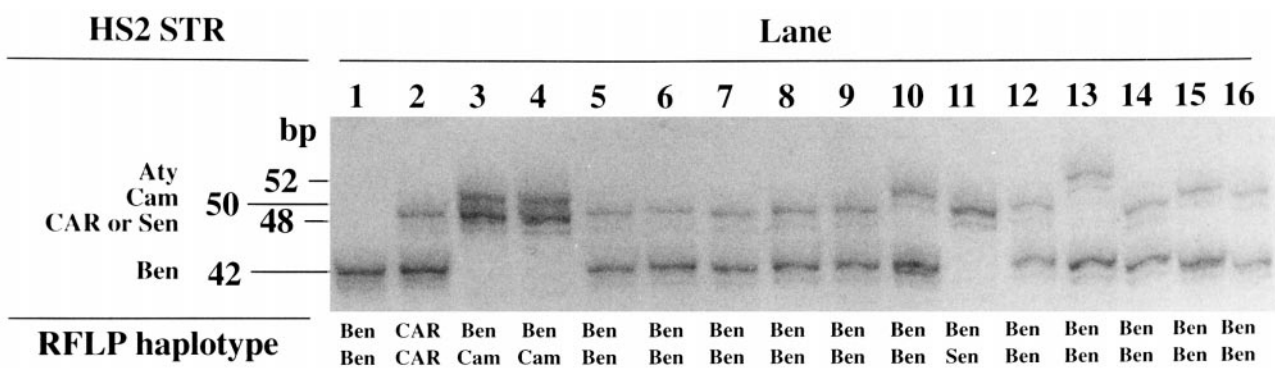

FIG. 2. Polyacrylamide gel of HS2 STR. Allele size in bp is shown. The 42 bp HS2 STR characteristic of the chromosomes with the Benin REFLP haplotype is shown in lane 1. The corresponding RFLP haplotype is shown in each lane: Ben = Benin, CAR = Central African Republic, Cam = Cameroon, Senegal = Senegal and Aty = Atypical.

TABLE 1

RFLP and HS2 STR Polymorphism in 200 β^S Chromosomes

RFLP haplotype	BENIN	CAR	HS2 STR SENEGAL	CAMEROON	ATYPICAL
BENIN	158	3	7	3	1
CAR	1	10	0	0	0
SENEGAL	0	0	7	0	0
CAMEROON	0	0	0	5	0
ATYPICAL	0	0	0	0	5[#]

Note. Concordance was assumed where alleles were heterozygous at both sites.
[#] Three distinct atypical haplotypes were identified.

signed using this strategy (Table 1). PCR-SSCP analysis confirmed the presence of a Senegal HS2 STR allele on one chromosome in a total of six patients (Fig. 4 lanes A-F), including two sib-pairs, homozygous for the Benin RFLP haplotype. The level of HbF in these patients ranged from 2.6 to 8.5% (Table 2) and the mean of 4.7% (n = 6) was not significantly different $p = 0.5$ from the mean of 5.8% for a control group of Benin homozygotes with concordant HS2 alleles on both chromosomes (n = 32). Four of the six patients have a normal α-globin genotype ($\alpha\alpha/\alpha\alpha$) and two a sib-pair (patients. C and D) heterozygous for α^+ thalassemia due to the 3.7 kb deletion (Table 2). This excludes possible influence of α-globin gene number on HbF level in these patients.

PCR-SSCP analysis was successfully applied in a novel manner to distinguish haplotypic variation of nucleotides −1450, −1280 and −1225 within the pre-$^G\gamma$ framework (Fig. 1). Polymorphic haplotypes for control Senegal and Benin chromosomes were confirmed by DNA sequencing. SSCP banding patterns for these and patient samples are shown in Fig. 4 (lower panel). In all six patients with hybrid Senegal-Benin chromosomes the pre-$^G\gamma$ framework is of the Benin type indicating the point of recombination for each of the variant chromosomes lies upstream of this element.

DISCUSSION

The contribution of *cis*-active elements to HbF level is central to understanding the variable clinical phenotype of SCA (41-43). Functionally important regulatory elements and their cognate binding proteins rep-

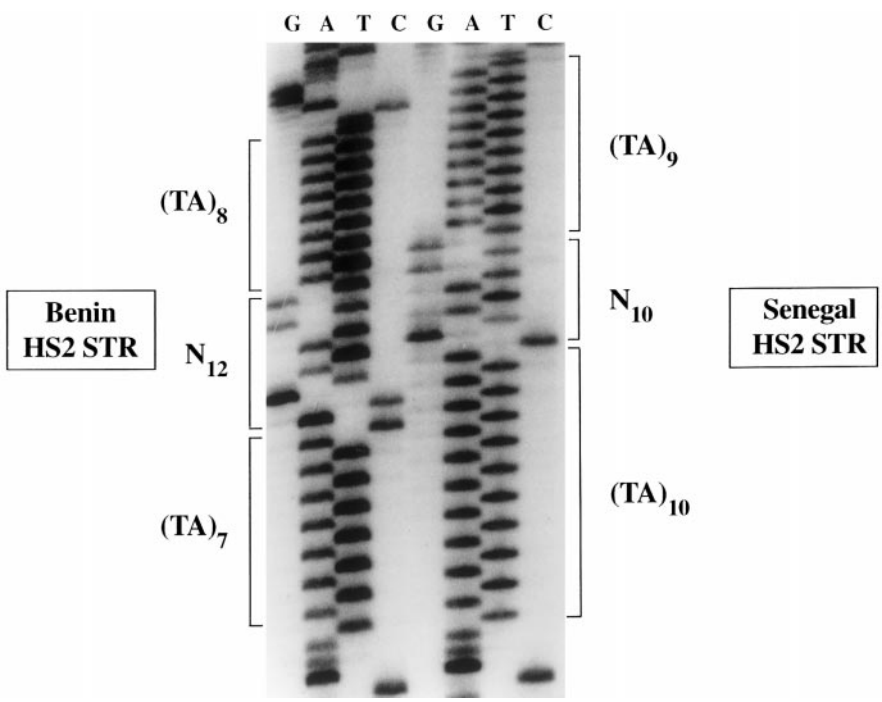

FIG. 3. Sequence of HS2 STR alleles linked to Benin (left) and Senegal (right) RFLP haplotypes. The sequence is of T-vector cloned PCR product amplified from genomic DNA of patient F (Table 2) known to be homozygous for the Benin RFLP haplotype.

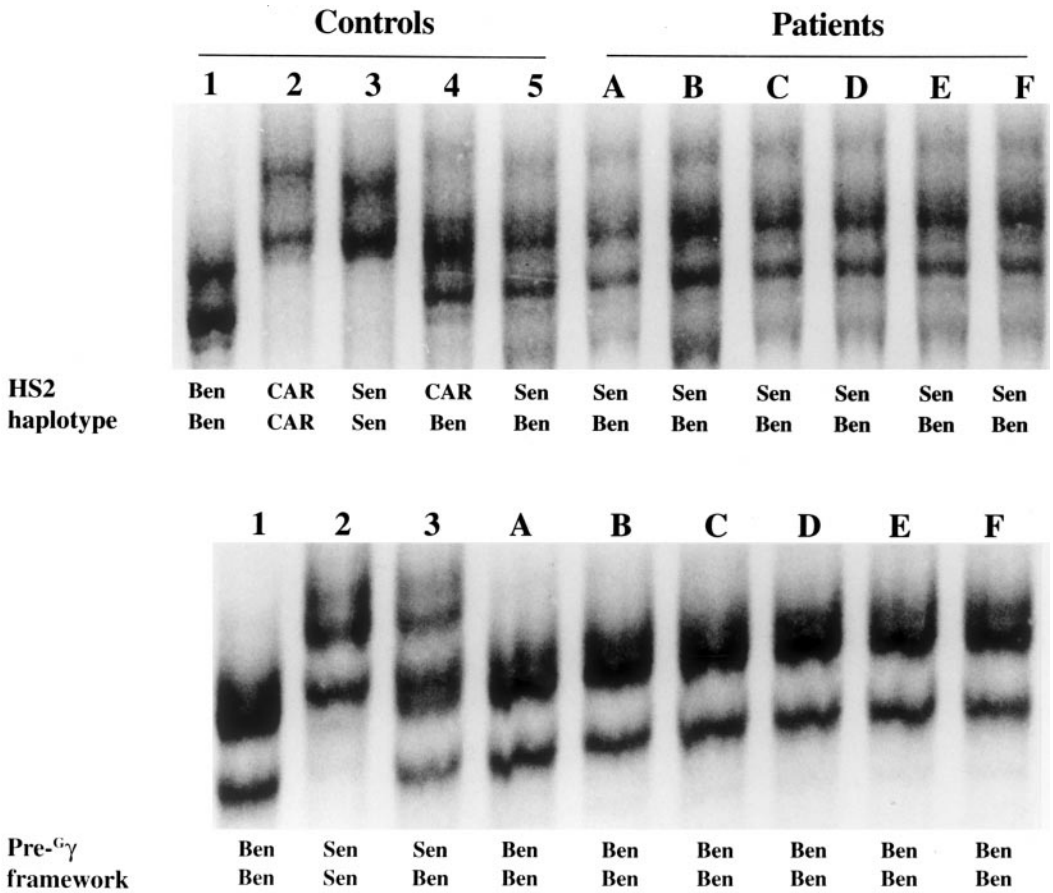

FIG. 4. PCR-SSCP analysis. Upper panel: Band shifts for HS2 STR alleles. Control samples are shown in lanes 1-5 and subseqent lanes (A-F) show the banding patterns for the patients. Lower panel: SSCP band shifts for pre$^{G}\gamma$ framework polymorphisms. Control samples are shown in lanes 1-3 and subsequent lanes (A-F) correspond to the patient samples as shown in the uper panel. The haplotypic associations for HS2 and pre$^{G}\gamma$ framework are shown under each lane (see Fig. 2 for abbreviations).

resent potential targets for pharmacological manipulation of globin gene expression and as potential utility in the treatment of β-globin disorders. The significance of a regulatory element is illuminated by the effect polymorphism within it have on HbF level. Variant chromosomes which have arisen probably by recombination and thus possess polymorphisms associated with both low and high HbF β^{S} chromosomes provide an opportunity to dissect the influence of individual *cis* elements on HbF level. The frequency of such variant chromosomes has not previously been systematically studied.

TABLE 2

Data on Patients with "Hybrid" Haplotype β^{S} Chromosomes with Senegal HS2 Allele Juztaposed to Benin β-Globin Gene Cluster RFLP Haplotype

Patient	Age (yrs)	Sex	HbF (%)	α-globin genotype
A	14	M	5.2	$\alpha\alpha/\alpha\alpha$
B	11	M	2.6	$\alpha\alpha/\alpha\alpha$
C	13	M	5.5	$-\alpha^{3.7}/\alpha\alpha$
D	13	M	4.0	$-\alpha^{3.7}/\alpha\alpha$
E	8	F	8.5	$\alpha\alpha/\alpha\alpha$
F	13	M	2.6	$\alpha\alpha/\alpha\alpha$

Note. Patients C, D and E, F are sibpairs.

This study of an unselected patient population using markers which span the interval between the LCR and expressed globin genes has identified several distinct variant chromosomes indicating that haplotypic diversity among β^{S} chromosomes is greater than hitherto appreciated (44, 45). Increasing evidence suggest that such atypical chromosomes arise probably as a result of recombinational events. It is unlikely that the Senegal-Benin chromosomes identified in this and previous studies resulted from mutations at the HS2 site on an ancestral Benin chromosome since such an event will require at least four separate mutational events. Significantly among chromosomes defined by a Benin RFLP pattern, the predominant β^{S} RFLP haplotype in the population studied, 14 of 172 (8.1%) chromosomes

had a non-Benin HS2 allele (Table 1). The most frequent haplotypic variation, juxtaposition of a Senegal HS2 allele with downstream Benin chromosomal elements, has previously been suggested to confer enhanced HbF production (32, 34). In contrast, all six patients with a Senegal-Benin chromosome detected in this study have a level of HbF within the range expected for the Benin haplotype. Thus, the presence of a Senegal HS2 allele does not *per se* consistently augment γ-globin gene expression from Benin chromosomes. In this respect it is of interest that deletion of HS2 in transgenic mice carrying yeast artificial chromosomes containing the entire functional human β-globin locus, results in minimally decreased globin gene expression at all stages of development (46). This implies functional redundancy within the LCR and makes it unlikely that polymorphism within HS2 alone would exert a marked effect on the pattern of globin gene expression.

The association between raised HbF or F-cell number and the Senegal HS2 allele has been observed in several individuals with a diverse ethnic, mutational and geographical background. Though unlikely, the possibility remains that the difference in HbF level observed between the patients in this study and other Benin RFLP homozygotes with Senegal HS2 polymorphism (33, 34) is due to a *trans*-acting mechanism. However, several independant observations of raised HbF in conjuction with the Senegal HS2 polymorphism suggest it is likely linked to downstream functional regulatory elements which either independently or in concert with HS2 upregulate γ-globin gene expression. On this basis the difference between the HbF level associated with Senegal-Benin chromosomes in this and previous studies can be reconciled by a model in which some variant chromosomes retain key *cis* elements linked to the Senegal RFLP haplotype that confer increased γ-globin gene expression, whereas in others, with a breakpoint upstream in closer proximity to the LCR, these elements are excluded. In keeping with this, the recombination site in the Senegal-Benin chromosomes identified by this study is located upstream of -1500 bp from the $^{G}\gamma$-globin gene cap site. This contrasts with the more distal breakpoint downstream from the LCR (between -369 and -309 bp from the $^{G}\gamma$-globin gene cap site) mapped in the two previously reported recombinants (32, 34). The level of HbF associated with individual Senegal-Benin chromosomes may therefore depend upon elements within the interval defined by the point of cross-over and the LCR.

Several putative functional regulatory elements upstream of the $^{G}\gamma$-globin gene which show haplotypic variation have been mapped. Haplotype specific DNA constructs which span from the $^{G}\gamma$-globin cap site to nucleotide -1227 fail to show any significant variation in expression in transient transfection assays (24). Differential enhancer function on the other hand has been attributed to *cis* motifs that show haplotypic variation within the pre-$^{G}\gamma$ framework (25). These include the CRE and a GATA-1 sites each of which is bound with increased affinity by their cognate proteins on Senegal sequences and by inference possesses greater enhancer activity relative to its Benin counterpart. The CRE and GATA motifs were of Benin-type on all six of the variant Senegal-Benin chromosomes characterised in this study. Whilst it remains speculative whether the pre-$^{G}\gamma$ framework or an as yet unidentified regulatory element dictates the differing γ-globin gene expression, no sequence variation between Senegal and Benin chromosomes upstream of this region has been described to date.

This study illustrates the value of hybrid haplotype β^{S} chromosomes in defining the contribution of *cis* active elements within the β-globin locus to γ-globin gene expression. The novel application of SSCP analysis described allows large numbers of chromosomes to be haplotyped rapidly. This will facilitate identification of other chromosomes which may illuminate the role of *cis*-active elements on the phenotypic expression of β-globin disorders. For example, it would be of considerable interest to know what HbF level results from chromosomes carrying a Benin HS2 allele and downstream Senegal elements, the reciprocal of those described here. This should resolve whether the combined effects of LCR and downstream *cis* elements or the latter alone are responsible for upregulation of γ-gene expression on Senegal chromosomes. This has important implications for the phenotypic variation of β globin disorders.

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