Hypersensitive site 4 of the human β globin locus control region

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Received January 28, 1991; Revised and Accepted March 5, 1991

ABSTRACT

The Locus Control Region (LCR) of the human β globin gene domain is defined by four erythroid-specific DNaseI hypersensitive sites (HSS) located upstream of this multigene cluster. The LCR confers copy number dependent high levels of erythroid specific expression to a linked transgene, independent of the site of integration. To assess the role of the individual hypersensitive sites of the LCR, we have localized HSS4 to a 280bp fragment that is functional both in murine erythroleukaemia (MEL) cells and in transgenic mice. This fragment coincides with the major area of hypersensitivity 'in vivo' and contains a number of DNaseI footprints. Bandshift analysis shows that these footprints correspond to binding sites for the erythroid specific proteins GATA1 and NF-E2 and a number of ubiquitous proteins, including jun/fos, Sp1 and TEF2.

INTRODUCTION

The human β globin gene locus consists of the embryonic ε globin gene, the foetal γ^G and γ^A genes, and the adult δ and β globin genes arranged in a 5′ to 3′ direction[1] (Fig. 1). A 5kb fragment containing the human β globin gene and flanking regions contains sufficient information to be expressed in a tissue and developmentally specific manner in transgenic mice[2,3,4], but expression levels are very low when compared to the endogenous mouse globin genes. Moreover, the transgenes are highly susceptible to position effects. A major breakthrough in overcoming this problem came from the study of a heterozygous Dutch γβ thalassaemia, which has a 100Kb deletion 5′ of the otherwise unaltered human β globin gene[5,6,7]. This allele is not expressed in the same cells that express the other (wild type) allele, suggesting that the sequences governing the expression of human β globin should be contained within the deletion. Four strong developmentally stable erythroid specific HSS upstream of the ε globin gene[8,9,10] were good candidates for such a control region. To test this possibility the four HSS were linked to a human β globin gene and shown to confer erythroid specific position independent expression on the transgene in mice at levels (per gene copy) comparable to the endogenous mouse globin genes[10]. The original construct, termed the minilocus, contained the four HSS in their natural configuration on a 20Kb fragment (Fig. 1). This was reduced to 6.5kb without losing the essential properties of the minilocus, by cloning the individual HSS on smaller restriction fragments[11] (Fig. 1, microlocus). This has allowed us to study the role of each HSS and to characterize the factors binding to each of the sites.

Stable transformation experiments with individual sites in MEL cells showed that the major activities were associated with HSS 3 and 2[12], the latter of which also shows activity in transient transfections[13]. Analysis in transgenic mice confirmed the properties of HSS 3 and 2, but showed that HSS 4 had a similar activity to HSS 2. HSS 1 showed very low levels of activity in both systems in agreement with the observation that a Spanish δβ thalassaemia[14,15] which has a deletion of HSS 2, 3 and 4 (retaining HSS 1), shows no β-globin gene activity.

We have previously studied HSS 2 and 3 in detail and shown that the functional activity of these sites is retained on fragments coinciding with the region that is hypersensitive in red cells[16,17]. In this paper we have used a similar approach to map the position of HSS 4 precisely, cloning it as a small fragment and testing its functional activity in MEL cells and transgenic mice. Full activity is retained on a 280bp fragment, which contains potential binding sites for the erythroid-specific factors GATA-1 and NF-E2 and for several ubiquitous factors including jun/fos, Sp1 and the CACC box binding protein TEF2. Binding sites for these factors also occur in HSS 2 and 3[16,17], but in a different spatial organization which may determine the absolute efficiency and/or developmental specificity of the individual HSS.

MATERIALS AND METHODS

Hypersensitive site mapping

Nuclei were isolated from the MEL clone 5 and L-cell population 200, both containing the minilocus[18] (Fig. 1B), as described previously[16]. DNA samples were cut with BamHI or XbaI, Southern blotted and hybridized with a 250bp BamHI-HaeIII fragment (BamHI digestions) or a 290bp BstNI-XbaI fragment (XbaI digests). The position of the HSS was estimated from the mobility of Sau3AI double digest fragments and radioactive λ-DNA digested with EcoRI and HindIII, or EcoRI, HindIII and BamHI.

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DNA constructs

Fig. 1D depicts the vector GSE 1273 containing the human β-globin gene as a 4.8kb BglII fragment and the neomycin-resistance gene as a selectable marker. The microlocus construct (GSE 1359) is as previously described[11]. The hypersensitive area of HSS 4 coincides with a 280bp SstI-AvaI fragment which was blunted and cloned in the HpaI site of GSE 1273[11] (Fig. 1D). For transfections, plasmids were linearized with PvuI. To generate transgenic mice, the SalI-EcoRV fragment containing the HSS and the human β-globin gene was isolated and blunted. Transgenic mice containing HSS 4 as a 2.1kb BamHI-XbaI fragment have been described previously[19].

Tissue culture and DNA transfections

The MEL cell line C88 was transfected by electroporation with 20μg of linearized plasmid DNA using a Bio-Rad Gene Pulsar set at 250 V, 960 μF. All other details are as described[20].

Transgenic mice

Constructs were dissolved at a concentration of 1−2μg/ml and injected into the male pronucleus of fertilized oocytes. 13.5 day foetuses were dissected and screened by Southern blotting using the second intron of the human β-globin gene to detect the transgene and the 3′ ApaI fragment of the endogenous mouse Thy-1 as a loading control. All transgenic foetuses were checked for mosaicism by comparing the signal of the transgene to that of the Thy-1 gene in DNA samples from body, yolk sac and placenta from the same foetus. RNA was analyzed by S1 nuclease protection as described[11].

DNaseI footprinting and band shift assays

The 280bp SstI-AvaI fragment was blunted and cloned in both orientations in the SmaI site of M13 mp8. Other details are as described previously[16], except that the band shift gels contained 0.5×TBE. The oligonucleotides used in this study are indicated in Fig. 4. Competitors were as described[16] or as shown in Fig. 4.

RESULTS

Fine mapping of hypersensitive site 4

Nuclei were isolated from the MEL clone 5 and the L-cell population 200, both of which have been stably transfected with the minilocus (Fig. 1) as described previously[18,21] and treated with different amounts of DNaseI. After purification, the DNA was digested with either BamHI or XbaI, Southern blotted and hybridized with the 250bp BamHI-HaeIII and the 290bp BstNI-XbaI fragments, respectively (Fig. 2). A clear area of hypersensitivity is found in erythroid cells only, at similar DNaseI concentrations as had been previously noted for HSS 2 and 3. There is no apparent change in HSS 4 after induction of MEL differentiation with DMSO (not shown). The same construct in non-erythroid L-cells does not display this hypersensitivity.

From comparisons with the mobility of Sau3AI double digest bands, we conclude that HSS 4 maps from 650 to 900bp from the 5′ BamHI site '*in vivo*' and these co-ordinates will be used henceforth (Fig.4). It is interesting to note that this area falls almost completely outside the fragment used to construct the so-called μLAR[22]. That particular μLAR fragment starts at the PstI site at 875 and overlaps only with the 3′ end of the hypersensitive area (Fig.4).

Functional analysis of the hypersensitive site

A 280bp SstI-AvaI fragment from 650 to 930bp 3′ to the BamHI site that overlaps with the area of hypersensitivity was blunted and cloned into the HpaI site at −800bp in the promoter of the human β-globin gene[16] (Fig. 1). This construct and a vector containing the full microlocus driving the β-globin gene[11] or only a β-globin gene were linearized with PvuI and three independent stably transfected MEL cell populations were generated for each construct by electroporation[20]. RNA was isolated from these populations four days after induction with DMSO and quantitated by S1 nuclease protection analysis (Fig. 3). The level of the human β-globin test gene with the HSS 4 deletion is considerably lower (approx 4%) than when driven by the full microlocus, but it is still much higher than the β globin gene without the HSS 4 (Table 1). The 4% level is similar to that obtained with the much longer 2.1kb BamHI-XbaI fragment driving the human β-globin gene[12].

An important feature of LCR elements is that they render expression of a linked gene independent from its site of integration in transgenic mice, hence expression levels follow the copy number of the transgene. Especially at low transcription levels this characteristic property of the LCR cannot be assayed easily in MEL cells; transfected cells are selected for G418 resistance, such that only integration events into active chromatin regions are observed.

We therefore used the HSS 4 deletion construct to generate transgenic mice. The construct described above was digested with SalI and EcoRV (Fig. 1), blunted, gel purified and injected into the male pronucleus of fertilized mouse eggs. Foetuses were analyzed 13.5 days after transfer of the eggs and transgenic foetuses were identified by Southern blots of EcoRI digested placenta DNA[10]; comparison of the transgene signal to that of the endogenous Thy-1 gene allowed an estimation of the transgene copy number. Each foetus was screened for mosaicism by comparing the Thy-1/transgene ratio in DNA from the body, yolk

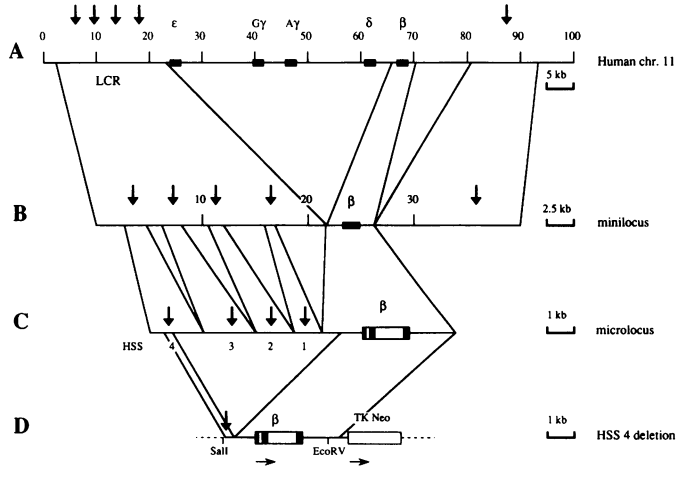

Fig. 1. The Locus Control Region of the human β-globin gene cluster. A) The human β-globin gene cluster on the short arm of chromosome 11. Four hypersensitive sites 5′ to the ε globin and one 3′ to the β-globin gene are indicated by vertical arrows. B) The 'minilocus' described by Grosveld *et al.*[10] C) The 'microlocus' constructed by Talbot *et al.*[11] D) The plasmid construct used in this study. The HSS 4 fragment was cloned in the HpaI site at −800bp. The SalI and EcoRV sites were used to isolate the fragment for injection in fertilized mouse eggs.

sac and placenta from the same animal. Using these criteria, one of the five transgenic offspring was highly mosaic (Table 1).

S1 analysis of the transgenic mice is presented in Fig. 3. We note that the animal with the highest copy number (no. 23) was anaemic, due to chain imbalance caused by overproduction of human β-chains[10,11,23]. The average expression level per gene copy of mice nos. 9, 19, 23 and 25 is similar to published data for mice with the 2.1kb HSS 4 fragment[19] (Table 1). The only exception is mouse no. 15, which does not express significant amounts of human β-globin mRNA. This is explained by the fact that the transgene could not be detected in body DNA, although both the placenta and the yolk sac were positive. Therefore, this foetus is mosaic with a very low, if any, contribution of the transgene to the haemapoietic system.

In conclusion, we have shown that the 280bp SstI-AvaI fragment, overlapping with the hypersensitive area of site 4, is the functional part of the 2.1kb fragment used in the microlocus[11]. Although it does not confer a high level of expression to a linked β-globin gene in MEL cells, it is capable of conferring integration site independent, copy number dependent expression in transgenic mice at high levels.

Protein DNA interactions in the 280bp HSS 4 fragment

Next, we determined protein binding to the functional element of HSS 4 by DNaseI footprinting *in vitro*. Fig. 4 shows the footprints obtained with MEL and HeLa extracts and we find two footprints, flanking a number of strong hypersensitive sites which are found with the erythroid extract, but not with the HeLa cell extract. Six other footprinted areas are found with both extracts, although some of these are weak. No differences were found between MEL, foetal liver and adult anaemic spleen extracts (not shown).

Based on the DNaseI footprinting we designed a number of oligonucleotides covering the footprinted areas and used these in bandshift experiments (Fig. 5). Oligonucleotide 870 is a double GATA-1 binding site, since the shifted products are efficiently competed by the −200 GATA-1 site of the human β-globin gene[24]. This is in agreement with the footprinting data (Fig. 4), since binding of GATA-1 is accompanied by the appearance of

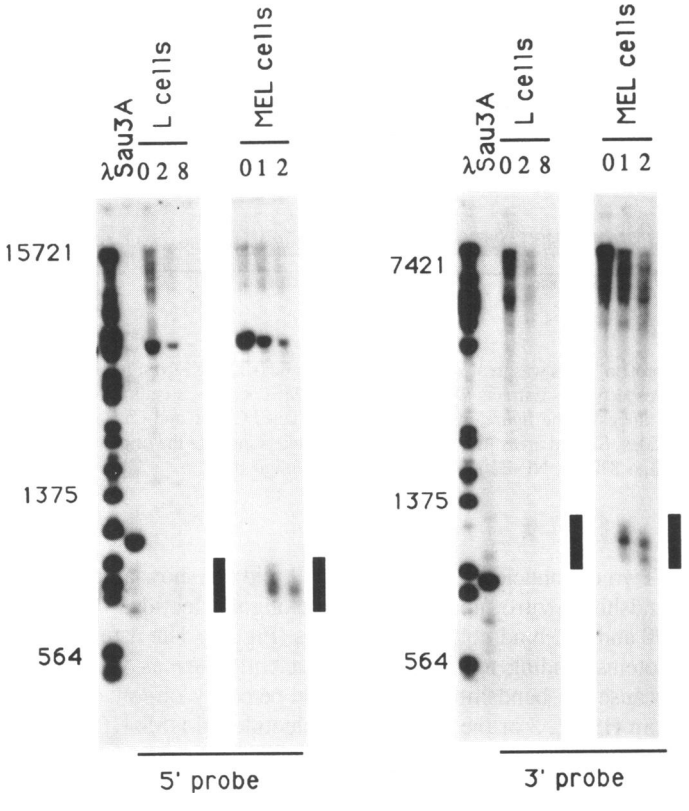

Fig. 2. Fine mapping of HSS 4 in MEL and L cells. Nuclei were isolated from MEL cells or L cells containing the minilocus[18]. DNA from DNaseI treated nuclei was digested with BamHI (left panel) or XbaI (right panel) and Southern blotted. A double digestion of Sau3AI and BamHI or XbaI was used as an internal reference (lanes Sau3). The amount of DNaseI (μg/mg DNA) is indicated. Radiolabelled λ restriction fragments were used as markers. The black bar indicates the hypersensitive area. The left two panels were probed with the 5' BamHI-HaeIII fragment, the right two panels with the 3' BstNI-XbaI fragment.

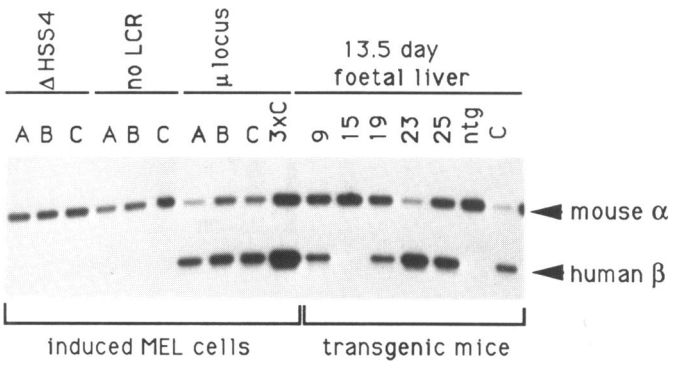

Fig. 3. Quantitative S1 analysis of the hypersensitive site 4. 5 μg of total RNA from induced MEL cell populations, or approximately 1μg of total 13.5 day foetal liver RNA was used per lane. 15μg total RNA from the microlocus population C (3×C) was included to monitor probe excess. Protected fragments, specific for the 5' ends of mouse α globin and human β-globin mRNA are indicated. ntg = non-transgenic control, C = control with 2.1kb BamHI-XbaI microlocus fragment[19]. Relative specific activity of the probes was Huβ : Mα = 1.47.

Table 1. Expression of the hypersensitive site 4 construct in MEL cells and transgenic mice. Bands were cut out of the gel and Cerenkov counted. A similar sized gel fragment just above the band of interest was also counted for background correction. The data given are corrected for the relative specific activities of the probes used. Copy numbers of the transgenic animals were determined from Southern blots. The copy number range in MEL cells was between 2 and 3 copies.

S1 analysis HSS4

MEL cells

Construct	Sample	Huβ/Mα	Average	% μ locus
Δ HS4	A	0.044	0.062	4
	B	0.067		
	C	0.074		
no LCR	A	0.005	0.004	0.3
	B	0.001		
	C	0.005		
Microlocus	A	1.624	1.542	100
	B	1.189		
	C	1.813		
	3×C	1.810		

Transgenic mice

Construct	Mouse	Copy no.	Huβ/Mα	Expr./copy
Δ HS 4	9	3	0.471	0.16
	15 mosaic	<1 in body	0.005	
	19	4	0.462	0.13
	23	35−40	4.147	0.12
	25	6	0.934	0.16
2.1kb HS4	157	13	1.877	0.14

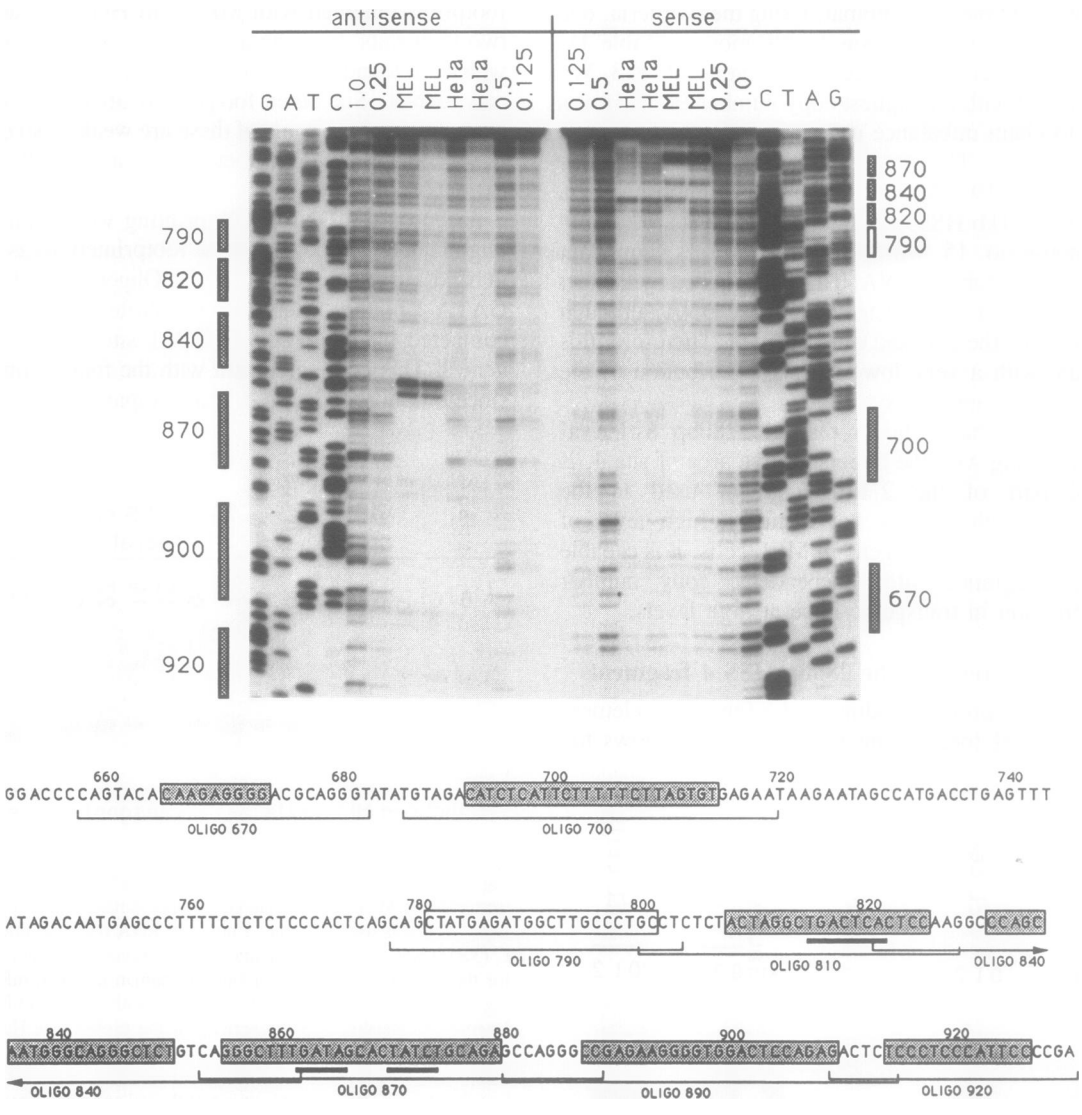

Fig. 4. DNaseI footprinting of the 280bp SstI-AvaI fragment. Top: The DNA in the 'no protein' lanes was treated with 1, 0.5, 0.25 and 0.125μg DNaseI as indicated. DNA was pre-incubated with 10 or 50μg protein and treated with 1 or 2μg DNaseI, respectively. Extracts were derived from MEL or HeLa cells as indicated. Both sense and antisense strands are shown. Footprinted regions are indicated by bars alongside the figure. A sequence ladder (lanes G,A,T and C) was used to map the footprints on the sequence. Bottom: Summary of the footprints and oligonucleotides. Shaded areas indicate footprints, brackets indicate the oligonucleotides used for bandshifts (Fig. 5). Black bars indicate consensus sequences for GATA-1 (oligo 870) or NF-E2/jun/fos binding sites (oligo 810).

a strong hypersensitive site on one side of the helix[24–27]. Interestingly, the 870 GATA-1 site contains two consensus binding sites in opposite orientation and from the bandshift competitions, we conclude that GATA-1 molecules can bind to both sites simultaneously (Fig. 5, 870 oligo, slower migrating complex). Similar arrays are found in HSS 2 and 3 of the human β-globin LCR[16,17] and in the chicken β-globin 3′ enhancer[26,27]. Interestingly the latter has recently been shown to direct copy number dependent expression of a linked transgene[28]. The 810 oligonucleotide contains a consensus for jun/fos/NF-E2[29] which has previously been shown to be important for the function of the PBGD promoter[30] and the β globin HSS 2[17,31]. As shown in Fig. 5, NF-E2 and jun/fos indeed bind to this sequence '*in vitro*' and we anticipate that the single site in HSS 4 acts as an enhancing element[17,31].

Two oligonucleotides, 670 and 790, did not show any specific bandshift in vitro (not shown), while oligonucleotides 700, 840, 890 and 920 bind ubiquitous proteins (Fig. 5). The nature of the proteins binding to the 700 oligonucleotide are as yet unclear because the bandshifts were not competed by oligonucleotides from HSS 2, 3 or the other oligonucleotides of HSS 4. The 840, 890 and 920 oligonucleotides are all capable of binding the general transcription factors Sp1[32] and the CACC box binding protein TEF-2[33] (Fig. 5). In addition, oligonucleotide 840 binds J-BP, a factor also found in HSS 2 and 3, as demonstrated by the competition for binding using the J-BP binding site from the HSS 2 (Fig. 5, panel 840). These general transcription factors may be required for proper functioning of HSS 4, contributing to its tissue-specific character via synergistic interactions with GATA-1 and NF-E2.

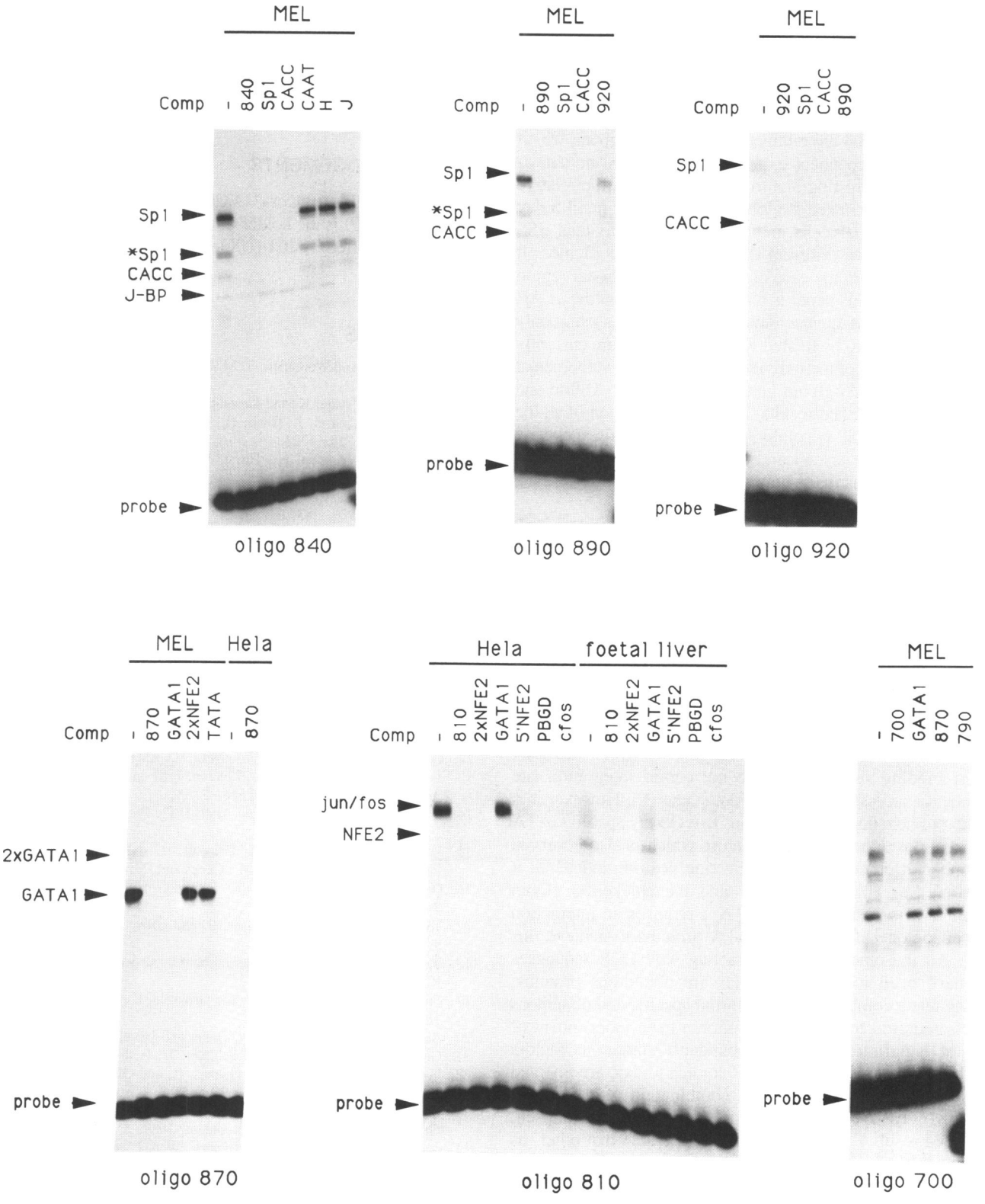

Fig. 5. Bandshift analysis of HSS 4. Oligonucleotide probes covering the footprinted areas (see Fig. 4) were used in gel retardation assays using MEL, foetal liver or HeLa extracts. Approximately 5μg extract was used per lane. Competitors were added as indicated in 100-fold molar excess prior to the addition of extract to the reaction. Sequences of the probes and competitors are given in Fig. 4 or have been described; − indicates no competitor. GATA1 competitor contains the −200 region of the human β-globin gene[24]; 2×NF-E2 is the HSS2 NF-E2 binding site[37]. 5'NF-E2 is the NF-E2 site of HSS 3[16]; PBGD & cfos are the NF-E2 binding sites from the PBGD & cfos gene promoters[29,30]; TATA competitor contains the β-globin gene TATA box[24]. Sp1 competitor[32], CAAT[24] and CACC box[16] competitors have been described. The H and J competitors contain binding sites for the H-BP and J-BP proteins of HSS2[17,37]. *Sp1 represents an Sp1-like factor and has been discussed previously[16]. The band running below J-BP in the panel with oligo 840 is not reproducible in every MEL extract and is most likely a degradation product.

DISCUSSION

In this paper we have delineated the functional element of HSS 4 of the human β-globin LCR. We demonstrate that it coincides with the major area of hypersensitivity in erythroid cells, as we have shown previously for HSS 2 and 3[16,17]. The basic properties of the HSS 4 are retained on a 280bp fragment, which confers position independent expression on a linked human β-globin gene. An interesting feature of HSS 4 is that the relative expression level of a linked β-globin gene is higher in 13.5 day foetal liver than in MEL cells, indicating that the individual HSS of the LCR may have different developmental specificities. It should be noted that in the case of HSS 4, the functional region we have mapped in this paper is virtually absent from the μLAR construct[22]. As a consequence developmental studies using only the μLAR may have to be treated with caution. We are currently investigating the role of the individual HSS in the developmental profile of the human β-globin gene cluster (Fraser, Dillon and Lindenbaum, unpublished results) and characterization of all the sequences involved will, hopefully, enable us to propose a concise model explaining the spatial and temporal expression patterns of the individual genes in the human β-globin gene cluster at the molecular level.

The proteins binding to the 280bp HSS 4 fragment show a high degree of similarity to those found interacting with the minimal fragments of HSS 2 and 3[16,17]. We have shown that the presence of binding sites for the erythroid (and megakaryocytic[34]) specific transcription factor NF-E2 is not sufficient for position independent expression of the transgene in mice in the case of HSS 2[17], although it is certainly required for the high levels of expression[17,31]. It is also known that although GATA1 is a transactivator[35], the presence of GATA-1 binding sites per se is insufficient to give position independent expression. The 5′ and 3′ regions of the human β globin gene contain at least six GATA-1 binding sites[24,25], but do not confer integration site independent expression on the β-globin gene[2,3,4]. However it is interesting to note that HSS 4 contains two closely spaced GATA 1 sites in opposite orientation, a feature which is also observed in a number of other elements conferring position independent expression, i.e. HSS 2[17] and 3[16] and the chicken β globin enhancer[28]. It is possible that GATA 1 requires an interaction with itself or one of the other GATA proteins to achieve this effect[36]. All the other factors interacting with LCR sequences which have been identified thus far are ubiquitous proteins, indicating that a combination of erythroid-specific and ubiquitous factors is required to render the β-globin gene independent of its site of integration[16,17]. The (abundant) ubiquitous factors shared by the three HSS of the LCR studied to date are Sp1 and TEF-2, but a simple multimerized combination of a GATA-1 and an Sp1/TEF-2 binding site is not functional (S.Philipsen, unpublished results). It is, therefore, conceivable that other, as yet less well characterised factors are involved, e.g. J-BP[37]. In addition, some factors may not be very abundant or soluble and could be overlooked in DNaseI and bandshift experiments with crude nuclear extracts. We are currently fractionating erythroid extracts to address at least the first part of this question.

The work described in this paper is one step further in the analysis of the β-globin LCR. Interestingly, similar sequences have recently been described for the human α-globin genes[38] and a detailed analysis of these sequences may aid our understanding of the regulation of these two gene families. Transcription from β-like and α-like gene clusters has to be activated co-ordinately throughout development and adult life to prevent chain imbalance. Since the LCRs play an important part in the absolute transcription level of individual genes and appear to be similar, they probably provide the key to understanding the coordinated expression of the genes from both clusters.

ACKNOWLEDGEMENTS

We are grateful to Cora O'Carroll for preparation of the manuscript. S.P. is the holder of a long term EMBO fellowship, D.W. is supported by ICI (UK). The work is supported by the MRC (UK).

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