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Function of the upstream hypersensitive sites of the chicken β-globin gene cluster in mice

Marc Reitman*, Eric Lee[1] and Heiner Westphal[1]

Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, Building 10, Room 8S-239 and [1]Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-1770, USA

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ABSTRACT

We have shown previously that the chicken β^A-globin gene, with its 3′ enhancer, is expressed in a copy number-dependent manner in transgenic mice. The expression level was low but increased ~6-fold upon inclusion of 11 kb of upstream DNA containing four DNase I hypersensitive sites. To study the effect of the individual upstream hypersensitive sites on transgene expression, we produced lines of mice in which the individual upstream sites were linked to the β^A gene and enhancer. RNA levels were measured in blood from adult animals. With each of these four constructs, the level of transgene RNA per DNA copy varied over a >20-fold range. These data suggest that addition of a hypersensitive site to the β^A-globin/enhancer region abrogates its position independent expression. The average β^A-globin expression per copy in the lines carrying an upstream site was comparable with that in lines without an upstream site. Thus, no single upstream hypersensitive site accounts for the higher level of β^A-globin expression seen in mice containing the complete upstream region. We had shown previously that control of the chicken β-globin cluster is distributed between at least two regions, the β^A/ϵ enhancer and the upstream region. Our current results suggest that the control mediated by the upstream DNA is itself distributed and is not due to a single hypersensitive site.

INTRODUCTION

The β-globin gene clusters provide an excellent opportunity to study the regulation of groups of related genes. The modern β-like globins in birds and mammals are believed to have evolved independently from a single gene present in their last common ancestor (1). Thus, it is not surprising that the organization of the chicken and human clusters is quite different. The human β-globin locus consists of five genes arranged in order of developmental expression and five upstream hypersensitive sites in ~65 kb of DNA (see 2). In isolation, the human globin genes are expressed variably and inefficiently in transgenic mice. However, when coupled to the upstream hypersensitive sites (3,4), the genes are expressed at a high level and in a copy-number dependent manner in transgenic mice (5). The upstream hypersensitive sites, constituting the locus control region (LCR), are believed to possess at least two activities. They are essential for the establishment of an active chromatin configuration (6,7) and they contribute to the developmental regulation of globin gene expression.

In the human cluster, the ability to mediate copy number-dependent expression is distributed redundantly among the upstream hypersensitive sites: all sites increased expression levels but the highest level of expression was obtained when all of the sites were included (8). Developmental stage-specific regulation of the β cluster is postulated to involve successive physical interactions between the upstream sites and the local regulatory regions of the individual genes (see 2,9–13 for reviews). Formation of a particular LCR-gene interaction depends on the proteins associated with the upstream sites and the genes, the order of the genes (14) and their distance from the LCR (15), and the lack of competing interactions (16).

The chicken cluster contains four genes that are not arranged in order of developmental expression (17). The embryonic genes (ρ and ∈) flank the fetal (β^H) and fetal/adult (β^A) genes (Fig. 1). A strong enhancer is found between the β^A and ∈ genes (18,19), and is required for transcription in reconstituted nuclei (20). Transient expression experiments suggested that developmental regulation of the β^A- and ∈-globins involved competition between their promoters for the enhancer (21,22), but this mechanism does not appear to be operative in transgenic mice (23).

The 11 kb upstream of the chicken globins contain four hypersensitive sites (24,25), as compared with five sites over 22 kb for the human cluster. The three chicken sites nearest to the genes are erythroid-specific sites, and two of them, 5′HS2 and 5′HS3, have moderate enhancer activity in a transient expression assay (26). The farthest upstream site, 5′HS4, is a constitutive hypersensitive site at one end of the globin chromatin domain (27,28), which can act as an insulator or boundary element (29) and does not have enhancer activity (26).

Studies of chicken β-globin cluster genes expressed in mice have produced some surprises. Mice carrying the β^A gene and 3′ enhancer without distant sequences, expressed the transgene in a

* To whom correspondence should be addressed

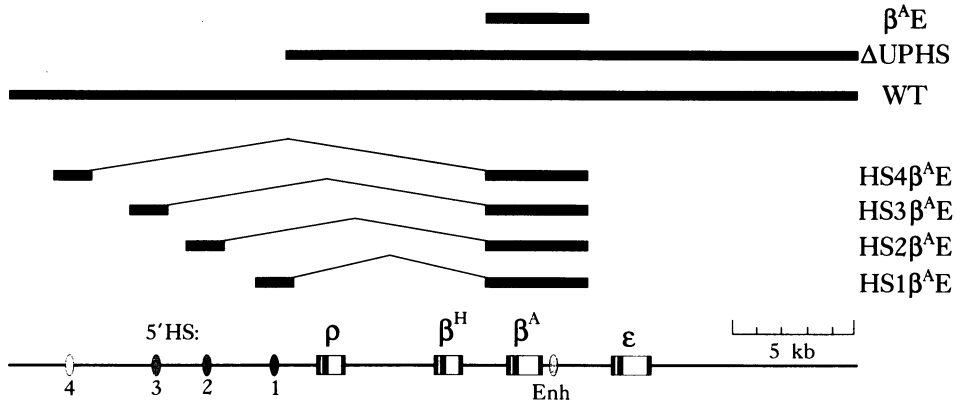

Figure 1. Constructs used to produce transgenic mice. The chicken β-globin cluster, with its ρ-, β^A-, β^A-, and ∈-globin genes (each transcribed from left to right), β^A/∈ enhancer (Enh), upstream hypersensitive sites (5'HS1–4), is shown at the bottom. Above are depicted the DNA fragments (β^AE, ΔUPHS, WT, HS1β^AE, HS2β^AE, HS3β^AE and HS4β^AE) used for making transgenic mice. The cluster and constructs are shown to scale.

copy number-dependent manner, albeit inefficiently (30). This demonstrated that position-independent expression could be mediated by sequences inside a cluster and near to a gene. The enhancer was required for β^A expression and both the promoter and enhancer were needed for chromatin opening (31). We have also studied mice carrying the whole chicken globin cluster. All four globins were expressed in an erythroid-specific and developmental stage-specific manner. Deletion of either the upstream hypersensitive sites or of the enhancer reduced the expression of the chicken globins, demonstrating distributed control of the cluster (32). We now examine the individual contribution of isolated upstream hypersensitive sites to globin expression.

MATERIALS AND METHODS

Transgenic mice

DNA for injection was obtained using standard cloning procedures (33). β^AE is a 4.45 kb *Eco*RI–*Bam*HI fragment containing the complete β^A-globin gene (marked with a 35 bp insert, AATTC-GAGCTCGGTACCCGGGGATCCTCTAGAGTC, in the *Rsa*I site of the 5′ UTR), the 3′ enhancer, and flanking DNA (34,30). The β^AE region was obtained from pUC18A′BC (34) which had been modified by insertion of a *Spe*I linker (5′-GGACTAGTCC) into the Klenow-blunted *Eco*RI site. The *Spe*I–*Sal*I β^AE fragment was inserted into *Spe*I–*Sal*I-cut pBluescriptIISK$^-$ (Stratagene) producing p791. Finally, the individual upstream hypersensitive sites, as *Xba*I cassettes of 1300–1700 bp (26), were placed into the *Spe*I site of p791. Plasmids of the 'A' type are defined as having the hypersensitive site and β^A-globin gene in their genomic orientation. Our laboratory designations for the plasmids with the A orientation are: HS1β^AE, p825; HS2β^AE, p818; HS3β^AE, p807 and HS4β^AE, p799.

DNA for microinjection was obtained free of vector sequences by *Not*I–*Sal*I digestion and gel purification. Transgenic FVB/N mice (35) were produced by microinjection into the male pronuclei (36) and screened by PCR on tail DNA (31). To eliminate the confounding effects of mosaicism and multiple integration sites, we used only progeny mice in these experiments.

DNA and RNA characterization

Southern blot analysis was performed as previously described (25). Transgene copy number was estimated using Southern and slot blots by comparison to spiked mouse DNA standards on the same blot (30). Transgene expression was quantitated by primer extension (30) or RNase protection (32) using RNA isolated from blood. The primers were: (i) 5′-GGTGATGAGCTGCTTCTCC-TC for chicken β^A-globin, giving extension products of 151, 154 and 156 nt on the marked gene, (ii) 5′-CAGGCAGC-CTTGATGTTGCTT for murine α-globin, with the major extension product of 65 nt, and (iii) 5′-TGATGTCTGTTTCTG-GGGTTGTG for murine β^{maj}-globin, with the major extension product of 53 nt. No difference in β^A:β^{maj} ratio was observed in *N*-acetylphenylhydrazine-treated, as compared to untreated, animals. DNase I hypersensitivity was assayed using nuclei from erythroid (spleen) and non-erythroid (liver) cells from phenylhydrazine-treated animals as described previously (31).

RESULTS

The current investigation into the function of the upstream hypersensitive sites was spurred by results suggesting that the region upstream of ρ-globin increased transgene expression. We had found that transgenic mice carrying the 4.5 kb β^AE construct expressed the β^A-globin gene at ~3% of the level of the endogenous β^{maj}-globin gene (30). Mice carrying a 24 kb fragment (ΔUPHS construct) containing all of the chicken β-like globin genes also expressed the β^A gene at this level. In contrast, in the context of the 35 kb complete chicken β-like cluster (WT construct), the β^A gene was expressed at a ~6-fold higher level (Fig. 1; Table 1; see reference 32). Our interpretation of these data is that the WT construct contains elements that increase the expression of the β^A gene and that these elements are not present in the ΔUPHS construct. Therefore, we now focus on the 11 kb upstream region that is present in the WT construct but absent from the ΔUPHS DNA.

The upstream region contains four DNase I hypersensitive sites that are likely to be the functional elements. We therefore studied the effect of the individual upstream sites on β^A expression in transgenic mice. Each hypersensitive site with flanking DNA totalling 1.3–1.7 kb was cloned upstream of the 4.5 kb β^AE

Table 1. Copy numbers and RNA expression levels

	Line	Copy Number	RNA level [A] mean	sd[B]	n[C]	RNA/copy[D]
β^AE	2674	1.5	1.6		1	2.1
	2671	3	3.1	0.2	3	1.9
	2673	7	8.3	1.6	6	2.6
	2681	7	12	0.7	4	3.3
	2702	8	8.3	2.1	3	2.2
	2680	37	61	21	4	3.3
ΔUPHS	ΔUPHS-2	1	1.1	0.4	2	2.2
	ΔUPHS-3	1	1.6	0.4	2	3.2
	ΔUPHS-4	1	1.6		1	3.2
	ΔUPHS-5	7	7		1	2.0
	ΔUPHS-1	8	11	4.3	2	2.9
WT	WT-1	1	9.1	0.9	2	18
	WT-2	2	16	4.2	4	16
	WT-3	2	15	6.4	2	15
HS1β^AE	HS1-A	4	7.3	1.6	6	3.8
	HS1-B	4	3.2	1.5	3	1.6
	HS1-C	14	4.4		1	0.6
	HS1-D	15	0.9	0.2	3	0.1
HS2β^AE	HS2-A	11	20	3.9	4	3.8
	HS2-B	16	31	2.5	3	3.8
	HS2-C	28	5.0	4.2	4	0.4
	HS2-D	40	2.0	2.3	4	0.1
HS3β^AE	HS3-A	5	0.8	0.6	6	0.3
	HS3-B	6	1.0	1.3	2	0.3
	HS3-C	21	24	1.6	2	2.3
	HS3-D	45	1.3	1.8	3	0.1
HS4β^AE	HS4-A	1	0.8	0.2	2	1.5
	HS4-B	4	<0.012		4	<0.008
	HS4-C	10	63	4.5	3	12
	HS4-D	17	29	6.4	3	3.4
	HS4-E	38	80	8.2	3	4.2

[A]β^A-globin transgene RNA as a percent of the total mouse β^{maj}-globin level.
[B]Standard deviation; average deviation when $n = 2$.
[C]This is the number of independent progeny whose RNA level was quantitated. Duplicate assays on a single RNA sample were averaged and counted as one point.
[D]β^A-globin transgene expression, per copy, as a percent of the level of one mouse β^{maj}-globin gene [i.e., 100 × (mean RNA level/copy number) × 2]. The data for the β^AE, ΔUPHS and WT lines have been reported previously (30,32).

fragment. These inserts were purified from vector sequences, injected into fertilized eggs, and the resulting mice were screened for transgene DNA. The founder mice were bred to produce transgenic lines, which were used in the subsequent experiments. Transgene copy number was determined by hybridization to slot blots containing tail DNA and these results are shown in Table 1. In most cases the copy number ranged from 4 to 40 and only one line was single copy. Southern blots were used to screen for rearrangements of the transgenes. Most lines showed intact, unrearranged transgenes, although some had aberrant fragments accounting for a minority of the transgene copies (data not shown).

RNA levels were measured in blood of adult animals. To control for RNA integrity and to allow quantitation, the transgenic β^A-globin and endogenous β^{maj}- and α-globin levels were determined simultaneously. Data for some of the HS1β^AE, HS2β^AE and HS4β^AE founders and progeny are shown in Figure 2. The RNA levels in the progeny tended to be slightly higher than in the founder mice, consistent with mosaicism in some of the founders. To further analyze the level of β^A-globin RNA expression in the various lines, the amount of correctly initiated RNA was quantitated and these results are presented in Table 1. As expected, the lines showed great variation in their total levels of transgene RNA. The degree of biologic variation in transgene expression between different animals of the same line is reflected in the standard deviation. This variation was proportionately larger in the lower expressing lines.

To characterize the variation in transgene expression between different lines, each line's β^A-globin RNA level was normalized to its DNA copy number. As reported previously, for the β^AE, ΔUPHS and WT constructs, normalization resulted in a constant expression per copy. In contrast, transgene expression from the HS1β^AE, HS2β^AE, HS3β^AE and HS4β^AE constructs was not copy number dependent. Each of these four constructs showed a >20-fold range in expression per copy. Since the β^AE lines make the same RNA, we attribute the variability in RNA levels to alterations in the rate of transcription rather than in mRNA stability.

In transgenic lines carrying portions of the human α-globin cluster, it has been noted that expression per copy decreases with increasing copy number (37). Therefore, we looked for such a relationship in our mice. A correlation was not found for the lines carrying the HS3β^AE and HS4β^AE DNAs. While there is a suggestion of such a relationship for the HS1β^AE and HS2β^AE constructs, more transgenic lines are needed before one can decide whether expression per copy really decreases with increasing copy number in these constructs.

One transgenic line, HS4-B, did not express any detectable β^A-globin. This remained true even after increasing the sensitivity of the assays for transgene RNA (Table 1). Southern blotting showed that ~3 of the ~4 copies of the transgene were not rearranged. While one might expect that chromatin opening precedes transcription, we have not been able to separate these events (31). To see if these events are dissociated in the HS4-B line, we examined the transgene's chromatin structure by looking for erythroid-specific DNase I hypersensitive sites. No such sites were found. As a positive control, we detected erythroid hypersensitive sites at the transgene enhancer, β^A promoter, and HS4 in cells from the HS4-D line. These data suggest that the HS4-B transgene is constitutively inactive, possibly because of integration into an obligately closed or inactive chromatin region.

The expression data are summarized in Figure 3. It is clear that expression per copy in the lines carrying the HS1β^AE, HS2β^AE, HS3β^AE and HS4β^AE constructs is not greater than in the β^AE and ΔUPHS lines. Of the 17 lines of mice generated, only one, HS4-C, approached the level of expression seen in the three WT lines. The other four HS4β^AE lines showed RNA levels comparable with or lower than that in the β^AE and ΔUPHS lines. From these data, we conclude that no individual hypersensitive site accounts for the increased β^A-globin expression seen in the WT mice.

DISCUSSION

We demonstrated previously that the control of the chicken β-globin cluster is distributed between at least two regions, the β^A/ϵ enhancer within the cluster and the 11 kb of DNA upstream

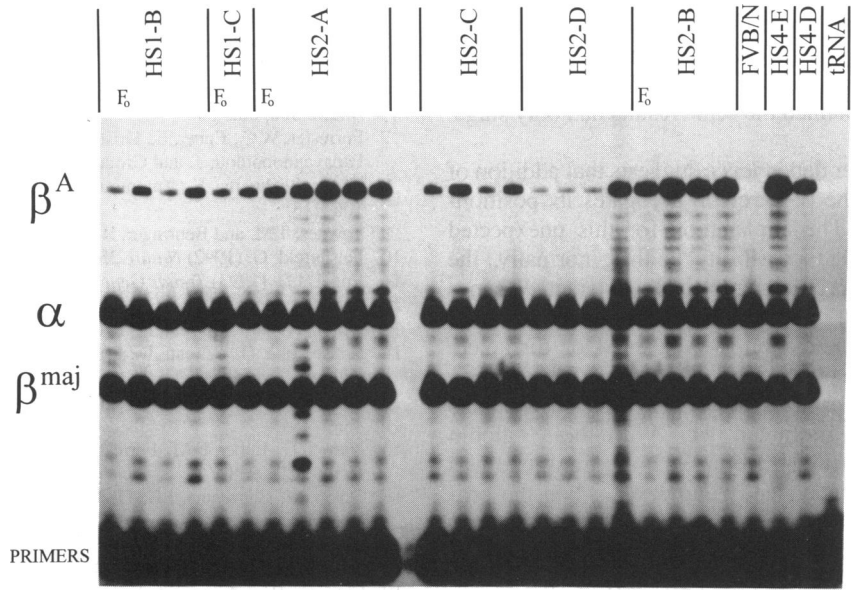

Figure 2. RNA levels in blood from transgenic mice. Primer extension was performed using 1 μg of RNA from the blood of adult animals. The source of the RNA is indicated at the top. When multiple samples are shown for a transgenic line, they are from independent progeny. F_0 denotes RNA from the founder, FVB/N is RNA from a non-transgenic mouse, and tRNA is a control substituting yeast RNA for blood RNA. Indicated at the left are the mobilities of the chicken β^A-globin transgene and endogenous mouse α- and β^{maj}-globin extension products.

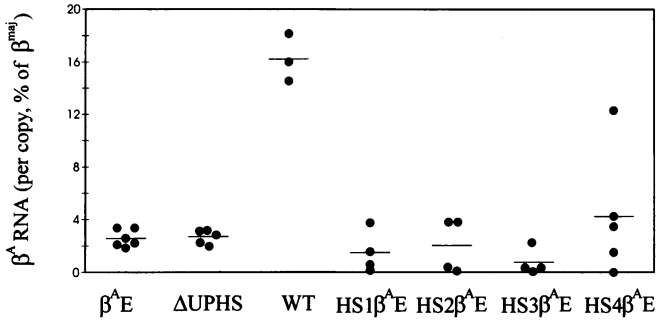

Figure 3. β^A-globin RNA level, per copy of the transgene. For each of the transgenic lines, the RNA expression per transgene copy per one copy of β^{maj}-globin is plotted. A horizontal line indicates the arithmetic mean for each construct.

of ρ. In those experiments, the complete upstream region increased β^A-globin expression ~6-fold. The current experiments assess the contribution of the individual upstream hypersensitive sites to β^A expression. Our main conclusion is that the addition of individual upstream hypersensitive sites to the chicken β^A-globin gene and its 3′ enhancer did not increase β^A expression in transgenic mice. Specifically, not one of the sites (irrespective of the presence or absence of enhancer activity as measured by transient expression) increased transgene expression in mice. These results suggest that the control mediated by the upstream region is itself distributed within this region and is not contributed solely or even predominantly by any one of the upstream hypersensitive sites.

Distributed control of gene expression has also been demonstrated for the lysozyme gene, which requires at least three regions for tissue-specific, copy number-dependent expression in transgenic mice (38). Thus single genes, as well as gene clusters, use multiple, dispersed control regions.

The individual upstream hypersensitive sites from the human β-globin cluster have also been studied. These sites show little sequence similarity to the chicken upstream sites, probably due to the small size of the functional regions and the large evolutionary distance between the classes (39). (We believe that the alternative, that the avian and mammalian upstream regions are not derived from common ancestral sequences and have unrelated functions, is less likely.) In the human cluster, any one of the four erythroid hypersensitive sites was able to confer copy number-dependence on the human β-globin gene, although in each case the level of transgene expression was lower than when all four erythroid sites were present. Some of the individual sites showed developmental specificity, with h5′HS3 best at stimulating γ-globin expression and h5′HS4 most selective for increasing β-globin expression (8). From the human data, one might have predicted that individual chicken hypersensitive sites would stimulate β^A-globin expression. However, the human cluster results are not directly comparable to ours since the basic chicken construct, β^AE, was expressed copy number dependently, while the human genes needed the upstream sites for copy number dependence. A direct comparison would require testing the chicken sites with a reporter, such as human β, that is not already expressed in a copy number-dependent manner.

As noted above, the functions of the hypersensitive sites depend on the assay used. By transient expression, two of the chicken upstream sites have enhancer activity, yet none stimulate expression in mice. In contrast, four of the human upstream sites stimulate expression in mice, but only one has much enhancer activity as measured by transient expression. These differences underscore the need to assay the function of putative control regions in distinct ways.

In the human cluster, multiple copies of individual upstream sites were needed for copy number-dependent expression (40). Our constructs containing individual upstream sites were predominantly multicopy and included the β^A/ϵ enhancer. It is not clear if we would have obtained the same results had only single copy lines been studied.

The loss of copy number dependence suggests that addition of a hypersensitive site to the β^AE region abrogates its position-independent expression. The explanation for this unexpected result is not obvious. One possibility is that, normally, the enhancer and β^A-globin promoter physically interact. When a normally distant upstream hypersensitive site is placed nearby, the enhancer–promoter interaction is weakened or disrupted, leaving the gene subject to position effects. A related observation has been made in mice carrying a μLCR plus different amounts of the human $^A\gamma$-globin promoter (41). With the $^A\gamma$-globin promoter extending downstream from –382, the transgene was expressed copy number dependently. However, when the promoter was extended to –730, copy number dependence was lost. The interpretation was that the –382 to –730 region disrupted the interaction between the LCR and the $^A\gamma$ promoter.

Comparisons between the chicken and human β-globin clusters continue to yield novel and illuminating results. The superficial similarities, with both loci consisting of a group of hypersensitive sites upstream of a group of genes, belie the differences in the details of how each achieves gene regulation. Competition appears to be important for the developmental regulation of the human genes, but experiments using transgenic mice suggest that competition may not play a major role in regulating the chicken genes. Copy number-dependent expression in the human cluster requires the upstream sites, while the chicken cluster can accomplish this with the β^A/ϵ enhancer. We have now shown that expression of the chicken β^A-globin gene is mediated by at least three distinct control elements, in addition to the gene itself. Our understanding of the chicken β-globin cluster should continue to help in elucidating the mechanistic details of gene regulation in other clusters.

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