Role of Upstream DNase I Hypersensitive Sites in the Regulation of Human α-Globin Gene Expression

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Erythroid-specific DNase1 hypersensitive sites have been identified at the promoters of the human α-like genes and within the region from 4 to 40 kb upstream of the gene cluster. One of these sites, HS -40, has been shown previously to be the major regulator of tissue-specific α-globin gene expression. We have now examined the function of other hypersensitive sites by studying the expression in mouse erythroleukemia (MEL) cells of various fragments containing these sites attached to HS -40 and an α-globin gene. High level expression of the α gene was observed in all cases. When clones of MEL cells bearing a single copy of the α-globin gene fragments were examined, expression levels were similar to those of the endogenous mouse α genes and similar to MEL cells bearing β gene constructs under the control of the β-globin locus control region. However, there was no evidence that the additional hypersensitive sites increased the level of expression or conferred copy number dependence on the expression of a linked α gene in MEL cells.

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Materials and Methods

DNA Fragments Used for Transfection

HS -40α. pXAJα-1.4, a 1.4-kb HindIII-EcoRI fragment including the HS -40 element attached to a 3.7-kb Bgl II-EcoRI fragment containing the human α1 globin gene has been described previously (Fig 1).

HS -40β. pRA1.4B was made by blunting the 1.4-kb HindIII-EcoRI HS -40 fragment and cloning it into an ApaI site in pBluescriptI (R.W. Jones, University of Oxford, Oxford, UK) that contains the entire human α1 globin gene in a 4.9-kb BglII fragment.

HS -40α. The construction of pAJ1.42α, in which a 2.2-kb EcoRI-BamHI fragment containing the human β2 gene was inserted between HS -40 and the α1 globin gene, has been described previously (Fig 1).

HS -40, -33α. To join HS -40 and HS -33, a recombinant [pAJ1.4(B)], containing the 1.4-kb HindIII-EcoRI fragment that includes HS -40, was linearized with BamHI; the ends were blunted with Klenow (Boehringer Mannheim, Lewes, UK) and phosphorylated. A fragment from a BamHI site in the polylinker of pUC13 to a genomic Tag I site spanning HS -33 was isolated from the recombinant pHinc -33/7 (a 2.0-kb HindIII-Tag I fragment spanning HS -33). This fragment was blunt and ligated into the modified BamHI site of pAJ1.4(B) to generate p -40, -33 with DNA corresponding to both HS -40 and HS -33 in their correct genomic orientation. A BamHI fragment containing HS -40 and HS -33 was isolated from p -40, -33 and inserted into the BamHI site of pXAJα, which includes the α1 gene, producing the recombinant pXAJα (-40, -33), with all segments in their correct genomic orientation (Fig 1).

4HSα. To join HS -10 and HS -8 to HS -40 and HS -33, p -40, -33 was linearized at a BamHI site that had reformed when the blunted Tag I site was ligated to the blunted BamHI site during its construction. Into this BamHI site a BamHI-BglII fragment
spanning HS -10 and HS -8 was inserted to produce p -40, -33, -10, -8. To join all four sites to the z and a genes, the insert from p -40, -33, -10, -8 was released with EcoRI and Sal I. The ends were then blunted with Klenow and Cla I linkers were added. The vector pXAJ 1.4z2a was partially digested with BamHI and Cla I linkers were added to create a unique Cla I site upstream of the z gene. After phosphatase treatment of this Cla I site, the fragment containing the four hypersensitive sites was cloned into the site producing the recombinant p cassette (Fig 1).

All plasmid fragments for transfection were released from the vector by Not I digestion and gel-purified before electroporation.

Transfection

A detailed description of the transfection procedure has been reported previously. Briefly, 1 µg pAPRT, containing the hamster adenine phosphoribosyl transferase gene, plus a 10-fold molar excess of each of the constructs was electroporated into 2 x 10⁷ cells, at which stage cells were harvested for RNA analysis and the remainder induced for 72 hours with hexamethylene-bisacetamide (HMBA) for RNA analysis. Individual clones of transfected cells were grown in methylcellulose, as described.

DNA and RNA Analysis

The copy numbers of the transfected fragments were estimated after Southern blotting, using the mouse erythropoietin gene as an internal standard for DNA concentration and a series of calibrated dilutions of p cassette z2a in total mouse DNA. To verify the uniqueness of individual clones, HindIII-digested DNA was hybridized with a 1.6-kb Pst I fragment containing the human a2 gene to detect junction fragments.

Individual clones positive for human DNA were identified by the polymerase chain reaction (PCR) using primers bracketing a 523-bp region of the HS -40 element. The 5' primer sequence was 5'-GAAAACGGAGCCGCTCCAGG-3' and the 3' primer was 5'-TGTTAGCTCCCCGAGGAGTTC-3'. Amplification was performed for 30 cycles, with denaturation at 94°C, annealing at 55°C, and extension at 72°C. RNA was analyzed by RNase protection assay and quantitated by counting bands cut from the gel.

RESULTS

a-Globin Gene Expression in MEL Cell Pools

Previous studies have shown that various DNA fragments containing HS -40 attached to an a-globin gene give high-level expression of the a-globin gene in MEL cells. We have now characterized this system in considerably more detail and have examined the levels of human a mRNA in pools of cells transfected with constructs containing HS -40 plus additional hypersensitive sites (Fig 1).

Figure 2A shows the levels of human and mouse globin gene expression in pools of MEL cells in a representative transfection experiment. Readily detectable levels of human a mRNA were observed in all of the pools after induction of hemoglobin synthesis with HMBA. However, there was marked variability from pool to pool in the expression of the human a genes relative to the endogenous mouse a genes (Table 1). In 29 pools transfected with the HS -40a construct, human a-globin mRNA ranged from 3% to 46% of the level of mouse a-globin mRNA. The copy number of the human genes in each pool was estimated from Southern blots of the DNA using copy number standards run on the same gel for comparison. The copy numbers also varied, but did not show a linear correlation with the expression levels. Indeed, the expression per gene copy was inversely correlated with copy number (Fig 3A). In three separate experiments, the mean expression per gene copy in 10, 10, and 9 pools was, respectively, 17.4%, 16.3%, and 42.8%, indicating quite a degree of variability, not only within an experiment but also between experiments.

Similar results were seen in MEL cell pools transfected with the HS -40zα, HS -40, -33α, and 4HSα constructs (Fig 2B and Table 1). Expression levels were within the range seen for the HS -40α construct and showed approximately fourfold variability from pool to pool. The mean expression per gene copy with the 4HSα construct was higher than with any of the other constructs. However, the
range of expression per copy was similar to that observed in the HS–40α experiments and the mean (49.4%) was not much higher than that seen in one of the HS–40α experiments (42.8%). When expression per copy was plotted versus copy number for the 4HSα construct, a similar inverse correlation was obtained (Fig 3B). Overall, therefore, we do not believe that these results provide any strong evidence that the additional hypersensitive sites significantly alter the levels of α-globin gene expression.

The levels of human α-globin mRNA expressed from the two constructs that also contained the human ζ gene were not significantly different from those that lacked the ζ gene. Expression of the ζ gene in the HS–40ζα construct has been reported previously16 and showed ζH/αH and ζH/αM RNA ratios of 12.4% and 2.1%, respectively; the mean ζH/αM/copy was 2.9%. With the additional hypersensitive sites in the 4HSζα construct, these values did not alter appreciably: ζH/αH = 8.5%; ζH/αM = 1.5%; and ζH/αM/copy = 3.3%.

With the HS–40β gene construct, copy numbers in the pools were rather higher, as were the levels of human β-globin mRNA relative to that of the endogenous mouse α mRNA. However, a similar relationship between expression per copy and copy number was observed (Fig 3C).

**α-Globin Gene Expression in MEL Cell Clones**

Given the variability in expression levels in MEL cell pools and the inverse relationship between expression per copy and copy numbers, the significance of any difference
in the mean expression per gene copy in Table 1 is difficult to gauge. Therefore, individual clones were obtained from pools containing the HS -40α, 4HSα, and HS -40β constructs by picking individual colonies of cells grown in methylcellulose. Positive clones were identified by PCR using primers specific for human HS -40. Approximately 50% of the clones contained the human DNA insert and the copy number in each clone was determined by Southern blot analysis and related to the RNA expression level. (Only copy number in each clone was determined by Southern analysis. (Only copy number per positive clone, differential growth of clones within a pool, copy number per positive clone, and inducibility of hemoglobin synthesis of individual clones after HMBA treatment. Thus, a pool made up of a majority of low copy number-positive clones could appear very similar on DNA analysis to one that contained mainly insert-negative clones together with a fraction of high copy number, insert-positive clones. However, given the exponential negative correlation between copy number and expression per copy, the expression levels in these two cases could be very different. Some of this variability results from the use of a cotransformation system. However, to use a linked selectable marker results in selection for positive position effects with the potential for biasing results. Based on our experience, we feel that this stable cotransfection system, although suitable for detecting major differences in expression between different constructs, may lack sensitivity for detecting small differences.

Although many of these variables should be evened out by looking at a large number of pools with a high clone content, the data clearly need to be interpreted with caution. The mean and range of human α mRNA levels in pools bearing the HS -40, -33α and 4HSα constructs did not differ from those containing only the HS -40α fragment. The mean expression levels per copy number were higher in these cases, but that could have been because they had lower

**Table 1. Expression of the Human α-Globin Genes in Pools of MEL Cells Transfected With Various Constructs**

<table>
<thead>
<tr>
<th>Construct</th>
<th>No. of Pools</th>
<th>No. of Clones Per Pool</th>
<th>Copy No. Mean</th>
<th>Copy No. Range</th>
<th>% αα M RNA Mean</th>
<th>% αα mRNA/Gene Copy Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS -40α</td>
<td>29</td>
<td>3-23</td>
<td>5</td>
<td>1-16</td>
<td>20.0</td>
<td>3-46</td>
</tr>
<tr>
<td>HS -40β</td>
<td>8</td>
<td>14-24</td>
<td>5</td>
<td>1-16</td>
<td>12.4</td>
<td>5-26</td>
</tr>
<tr>
<td>HS -40, -33α</td>
<td>10</td>
<td>6-23</td>
<td>4</td>
<td>1-8</td>
<td>27.7</td>
<td>11-43</td>
</tr>
<tr>
<td>4HSα</td>
<td>10</td>
<td>2-12</td>
<td>2</td>
<td>1-8</td>
<td>21.3</td>
<td>11-40</td>
</tr>
<tr>
<td>HS -40β</td>
<td>8</td>
<td>36-81</td>
<td>11</td>
<td>8-16</td>
<td>67.0</td>
<td>45-91</td>
</tr>
</tbody>
</table>

DISCUSSION

MEL cells, stably transfected with human globin genes, have been widely used as an experimental system for the analysis of globin gene expression and the role of upstream regulatory elements on the level of gene transcription. Thus, with the human β-globin gene complex, dissection of the four β-LCR hypersensitive sites was performed by transfecting various combinations of these sites attached to a β-globin gene and analyzing their expression in MEL cells, before confirming the observations in transgenic mice.15,19,20 In this way, the predominance of hypersensitive sites 2 and 3 in the MEL system was shown. We have used this same approach to characterize the region upstream of the human α-globin complex with respect to enhancing expression of the α-like globin genes.

We have observed some variability between replicate pools. Therefore, to compare the levels of α-gene expression from different constructs, we have examined a much larger number of pools and clones of transfected cells than had previous investigators. This variability, seen both within and between experiments, is likely to be due to a number of sources such as transfection efficiency, proportion of APRT-positive clones carrying the insert, differential growth of clones within a pool, copy number per positive clone, and inducibility of hemoglobin synthesis of individual clones after HMBA treatment. Thus, a pool made up of a majority of low copy number-positive clones could appear very similar on DNA analysis to one that contained mainly insert-negative clones together with a fraction of high copy number, insert-positive clones. However, given the exponential negative correlation between copy number and expression per copy, the expression levels in these two cases could be very different. Some of this variability results from the use of a cotransformation system. However, to use a linked selectable marker results in selection for positive position effects with the potential for biasing results. Based on our experience, we feel that this stable cotransfection system, although suitable for detecting major differences in expression between different constructs, may lack sensitivity for detecting small differences.

Fig 3. The relationship between expression per gene copy and copy number for pools of MEL cells containing the (A) HS -40α, (B) 4HSα, and (C) HS -40β constructs.
mean copy numbers. It should be noted that there is a broad range of expression per gene copy with all of these constructs and the differences in the means are not significant.

A more reliable measure of the expression levels of different constructs should be obtained by comparing individual clones, at equivalent copy numbers. The clonal data presented here confirm the inverse correlation between expression per copy and copy number that was observed in the pools. However, when these data are plotted out for the different constructs, no difference in human α mRNA per copy is observed between cells containing fragments that only include the HS −40 and those with the 4HSα construct, which contains in addition the hypersensitive sites at −33, −10, and −8 (Fig 4). Thus, in MEL cells, the −33, −10, and −8 hypersensitive sites are not only incapable of activating α-globin gene expression in the absence of HS −40,1,6 but also do not appear to synergize with HS −40 in this function. Also, they do not appear to affect the level of α-gene expression in this system.

The level of human α mRNA in single-copy clones containing HS −40 is approximately equivalent to that of each of the endogenous mouse α genes, irrespective of whether α, β, or β genes are attached to it (Fig 4). It is unlikely that small, but perhaps significant, differences in expression levels would be detected by this system, but preliminary evidence from lines of transgenic mice also indicates that the additional hypersensitive sites do not increase the level of α-globin RNA over those containing HS −40 alone (unpublished observation). The results reported here are similar to those obtained in cells bearing the intact β-LCR attached to a β-globin gene, where, again, at low copy number, the expression of the human gene is more or less equal to that of the endogenous globin genes.12,17,19,20 In contrast, when individual hypersensitive site elements of the β-LCR are used, lower expression levels are observed.12,17 However, we cannot conclude that HS −40 is the equivalent of the β-LCR until the differences in copy number dependence and the stability of expression during development in transgenic mice have been resolved.7 It may be that HS −40 is only sufficient for α-globin gene regulation in the wider chromosomal context of the entire α-gene cluster and that differences in the chromosomal structure of the two clusters may underlie the differences observed in expression systems.21 However, the roles of the additional hypersensitive sites at co-ordinates −8, −10, and −33 kb remain to be determined.

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