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The β-globin locus control region enhances transcription of but does not confer position-independent expression onto the *lacZ* gene in transgenic mice

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The β-globin locus control region (LCR) confers high levels of position-independent, copy number-dependent expression onto globin transgenes. Here >40 independent transgenic mouse lines and founders that carried the LCR *in cis* with the β-globin gene promoter driving a *lacZ* reporter gene were studied. Expression of the *lacZ* transgene was assayed by measuring β-galactosidase enzyme activity in fetal liver extracts, the levels of which correlated with the quantity of *lacZ* mRNA determined using RNase protection assays. Unexpectedly, expression of the *lacZ* transgene was found to show strong position effects, varying as much as 700-fold per transgene copy. These position effects occurred even if the whole β-globin gene was incorporated as part of the *lacZ* reporter gene. Moreover, DNase I-hypersensitive sites appeared in the transgene LCR in high expressing but not in low expressing lines, suggesting that the LCR itself was position dependent. In contrast, MEL cell clones, in which transcriptionally active integration sites were selected for, gave <13-fold variation in expression per copy of an LCR–*lacZ* construct. These results show that the *lacZ* reporter affects the ability of the LCR to activate chromatin in mice and that culture cells are not an adequate model for position-independent gene expression studies.

Keywords: β-globin LCR/*lacZ*/position independence/transgenic mice

Introduction

The human β-globin gene locus contains five active genes that are arranged in their developmental order of expression (Figure 1A). ε-Globin is expressed only during erythropoiesis in the yolk sac of the early embryo, the two γ-globins are expressed at high levels in the red blood cells derived from the fetal liver, while δ-globin, a minor contributor, and β-globin are expressed during erythropoiesis in the bone marrow late in fetal life and throughout adult life (reviewed in Stamatoyannopoulos and Nienhuis, 1994). To express at high levels, these genes rely on the activity of a locus control region (LCR). The human β-globin LCR, which is located 6–18 kb upstream from the ε-globin gene (Figure 1A), is characterized by four strong DNase I-hypersensitive site (HSS) regions that appear specifically in nuclei of erythroid cells (Tuan *et al.*, 1985; Forrester *et al.*, 1986).

The β-globin LCR has two important properties associated with it: a very strong erythroid-specific enhancer activity and the ability to confer position-independent, copy number-dependent expression onto globin genes under its control (reviewed in Townes and Behringer, 1990; Epner *et al.*, 1992; Dillon and Grosveld, 1993). Although it is still unclear how this latter property functions, it is sometimes referred to as chromatin opening activity. Globin transgenes that include the LCR are expressed in all independent transgenic mouse lines and erythroid cell clones (Grosveld *et al.*, 1987; Talbot *et al.*, 1989). The levels of expression are comparable with that of the endogenous globin genes and increase in proportion to the number of copies of the transgene present in each line, independently of where the transgene integrates into the genome. In contrast, globin transgenes without the LCR show strong position effects, which are characterized by very low and highly variable levels of expression with no relationship to copy number. In fact, 30–70% of mice harbouring globin transgenes without the LCR give no detectable expression (Chada *et al.*, 1985; Costantini *et al.*, 1985; Townes *et al.*, 1985). LCRs have also been described for a number of other gene loci (for examples, see Greaves *et al.*, 1989; Palmiter *et al.*, 1993; Bonifer *et al.*, 1994; Madisen *et al.*, 1994; Talbot *et al.*, 1994; May and Enver, 1995) and are expected to be essential for the correct expression of many genes.

Although only the HSS2 region of the β-globin LCR shows enhancer activity in transient assays, three of the four HSS regions (HSS2, HSS3 and HSS4) demonstrate enhancer activity in stably transfected erythroid culture cells and/or transgenic mice (Philipsen *et al.*, 1990; Talbot *et al.*, 1990; Pruzina *et al.*, 1991). The full enhancer activity of the LCR requires a combination of at least two and possibly all three of these HSS regions. DNA fragments containing each of the four HSS regions have been combined into a plasmid referred to as the microlocus cassette (Talbot *et al.*, 1989; Collis *et al.*, 1990). This microlocus retains full LCR enhancer activity and the ability to confer position-independent expression onto globin transgenes in mice.

The presence of DNase I-hypersensitive sites in LCRs may reflect whether or not the LCR is situated in transcriptionally active chromatin (Reitman *et al.*, 1993; Huber *et al.*, 1994) and/or whether it is bound by specific transcription factors (Stamatoyannopoulos *et al.*, 1995). With the chicken β-globin LCR *in cis* with a globin gene void of a promoter, DNase I hypersensitivity in the LCR was observed in only 60% of transgenic mouse lines (Reitman *et al.*, 1993). This was in contrast to 100% of mice showing DNase I-hypersensitive sites when the gene promoter was present. Thus, certain gene components may

be necessary for the LCR itself to be active. In murine erythroleukemia (MEL) cells, with the β-globin promoter driving a β-globin cDNA in the microlocus vector, only very low levels of expression were obtained (Collis *et al.*, 1990). Addition of more β-globin gene sequences, including the second intron, was necessary to restore high expression. This was attributed to increased transcription rates as well as increased mRNA stability. Therefore, it also appears that particular gene sequences and/or structures may be necessary for the LCR to enhance transcription. However, in studies with MEL cells it was not determined whether expression was also position independent. Hence, whether low expression with just a β-globin cDNA could also be due to an inability of the LCR to act in a position-independent manner is not known. Moreover, stably transfected populations and clones of MEL cells are selected with G418 for expression of the *neo* gene marker present in the microlocus plasmid (Talbot *et al.*, 1989; Collis *et al.*, 1990). Since the promoter of the *neo* gene must be transcriptionally active, one only selects cells in which this gene has been activated. Thus, cell culture systems may not be ideal for studies regarding position-independent gene expression.

Here it is shown that the β-globin LCR is incapable of conferring position-independent expression onto a *lacZ* reporter transgene in mice, even when the whole β-globin gene is included in the transcription unit. Yet, LCR enhancer activity in the *lacZ* gene-containing constructs is at least partially maintained in mice and the same LCR–*lacZ* constructs express at near position-independent, copy number-dependent levels in MEL cells. These results show unequivocally that MEL cells are not an adequate model for position-independent studies. The data are discussed in relation to previous results regarding the mechanism of LCR activation.

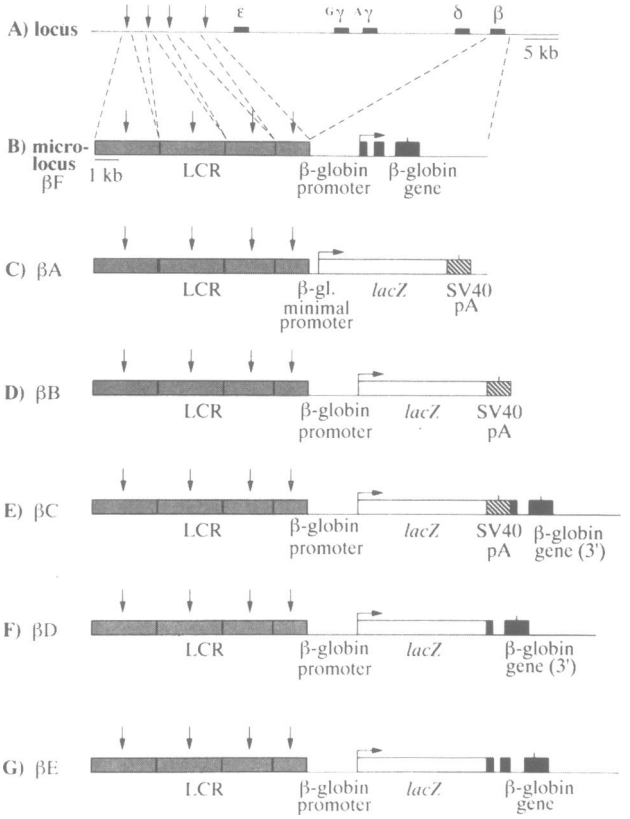

Fig. 1. Structure of the β-globin locus and microlocus constructs. (**A**) The structure of the β-globin locus with the position of HSS regions in the LCR indicated by arrows. (**B**) The structure of the β-globin microlocus designed by Collis *et al.* (1990). Black boxes indicate the three exons of the β-globin gene. (**C–G**) The structure of the various *lacZ* reporter constructs.

Results

The β-globin LCR does not confer position-independent expression onto a lacZ reporter transgene

In the transgenic mice studies described below, the β-globin gene promoter from –800 to +32 [relative to the transcription start (Cap) site] or a β-globin promoter deleted to –87, containing only the TATA and CAAT box motifs of the minimal promoter, was used. The promoter fragments were linked to a *lacZ* reporter gene (which codes for the β-galactosidase enzyme) and this was followed by SV40 polyadenylation signals. These reporter constructs were cloned into the microlocus cassette described by Collis *et al.* (1990). The final constructs, denoted βA and βB, are shown in Figure 1. Throughout the results, when we refer to LCR we mean as it exists in the microlocus cassette.

Five and seven stable transgenic lines for the βA and βB constructs, respectively, were established. Adult blood cells isolated from transgenic animals were incubated in the presence of X-gal to detect β-galactosidase activity *in situ*. Three of the five βA lines (βA2, βA4 and βA5) and all seven of the βB lines demonstrated blue colouration of some of the red blood cells in this assay. An example of this is shown in the top panel of Figure 2. The percentage of cells that coloured blue varied considerably between the different lines, although it varied very little between animals of the same transgenic origin. No red blood cells stained in two of the βA lines (βA1 and βA3) (not shown). When whole 15.5 day transgenic fetuses from the βA and βB lines were stained similarly *in situ*, the circulatory system and the fetal liver, the site of erythropoiesis at this stage of development, were coloured blue (Figure 2, middle and bottom panels). The intensity of staining of the circulatory system and fetal liver varied among the different transgenic lines, and the two βA lines (βA1 and βA3) that gave no detectable staining of adult red blood cells also gave no colouration at the fetal stages (not shown). No blue colouration was observed outside of the circulatory system and fetal liver for any of the βA and βB lines. Thus, expression of the *lacZ* reporter gene was erythroid specific, as would be expected for a β-globin LCR-driven transgene (Grosveld *et al.*, 1987; Blom van Assendelft *et al.*, 1989).

To determine the expression level per transgene copy, the copy number was quantified on Southern blots of genomic DNA (Figure 3) and β-galactosidase activity was measured in extracts prepared from fetal liver. The results are summarized in Table I. The two βA lines that showed no staining (βA1 and βA3) gave no measurable β-galactosidase activity (Table I). In contrast, the other three βA lines gave relatively high β-galactosidase activity (βA2, βA4 and βA5 lines in Table I).

Four of the βB lines (βB3, βB4, βB5 and βB6) gave

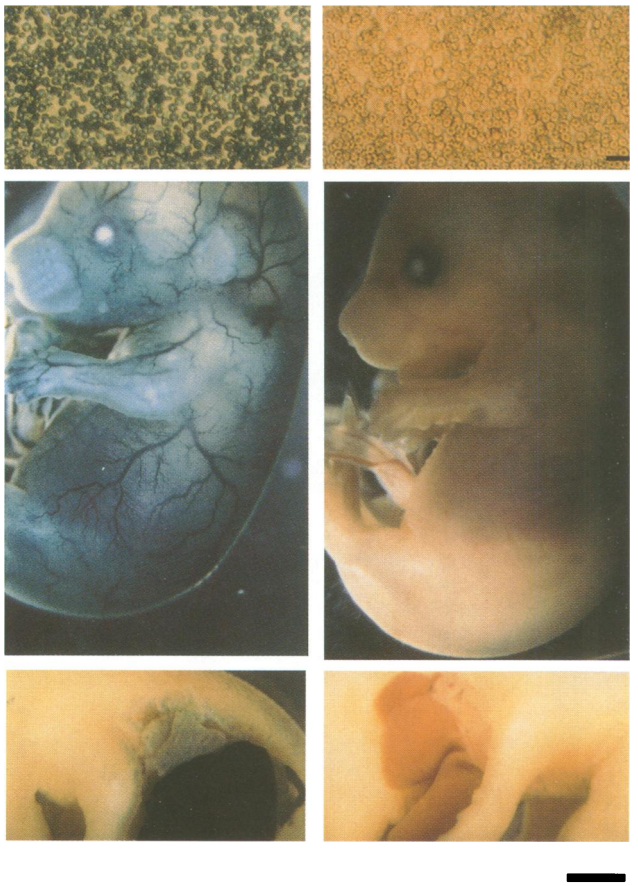

Fig. 2. *In situ* detection of β-galactosidase activity in adult red blood cells and fetal tissues. The top panel shows the tail blood from adult mice (transgenic line βB6), the middle panel shows 15.5 day fetuses (transgenic line βB1) and the bottom panel shows 15.5 day fetuses (transgenic line βA4) with the liver dissected out. The right of each panel is a non-transgenic litter mate. The scale bars in the top and bottom panel represent 20 and 1000 μm, respectively. The fetuses in the middle panel were incubated in X-gal solution overnight to stain the circulatory system, whereas the fetuses in the bottom panel were only incubated for 30 min.

an expression per transgene copy that was comparable with or greater than the activity seen with the expressing βA lines. On the other hand, three of the βB lines (βB1, βB2 and βB7) gave relatively low expression per transgene copy. Thus, for both the βA and βB lines, β-galactosidase activity levels were highly variable and did not correlate with transgene copy number.

To ensure that none of the lines had sustained deletions and/or rearrangements of the transgenes, a detailed DNA analysis was done for all of the βA and βB lines (data not shown). Genomic DNA was digested separately with *Sac*I and *Xba*I, which cut the transgene constructs into nine and three fragments, respectively. Southern blots of the digested DNA were hybridized in succession with a number of probes that cover the entire construct (not shown). All fragments of the correct size were detected in the lines included in the analyses. Taken together, these results suggest that the β-globin LCR does not confer position-independent expression onto the *lacZ* gene in mice, at least when only a β-globin promoter is present.

We also determined whether DNase I-hypersensitive sites were present in the transgene LCR of different transgenic lines. Hypersensitive sites in the transgene

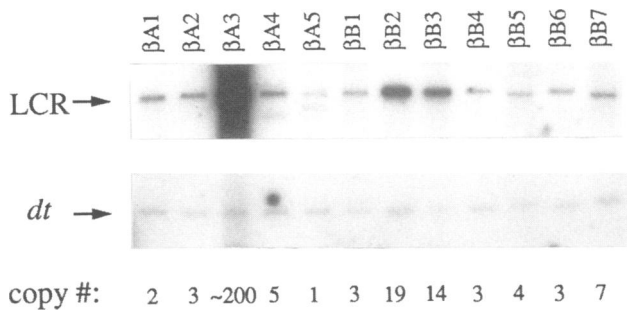

Fig. 3. Copy number determination. DNA from the transgenic mouse line shown at the top of each lane was digested with *Sac*I and then analysed by Southern blot hybridization. The membrane was hybridized with a DNA fragment from the LCR in the microlocus and with a control probe from the mouse *dt* locus. Copy number was calculated as the ratio of the LCR band intensity over the *dt* band intensity.

Table I. Analysis of βA and βB transgenic lines

Line	β-gal.[a] (mU/mg)	Copy no.[b]	Expression/copy (mU/mg)/copy
βA1	0	2	0
βA2	220	3	73
βA3	0	~200	0
βA4	270	5	54
βA5	60	1	60
βB1	25	3	8.3
βB2	25	19	1.3
βB3	590	14	42
βB4	720	3	240
βB5	610	4	152
βB6	900	3	300
βB7	8	7	1.1

[a]Extract from fetal liver was assayed for β-galactosidase activity.
[b]Copy number was determined using F1 tail DNA.

human β-globin LCR were detected in fetal liver nuclei isolated from two high expressing lines (βB5 and βB6; Figure 4A and B) but were not detectable in nuclei isolated from two low expressing lines (βB1 and βB7; Figure 4C and D). As a positive control, DNase I-hypersensitive sites in the endogenous mouse β-globin LCR were detected in these latter two cases using the same DNA (Figure 4E and F). Therefore, in terms of hypersensitive sites, the LCR itself was demonstrating position effects in these LCR–*lacZ* transgenic mice.

Addition of the whole β-globin gene does not restore position-independent expression to the lacZ reporter

As it was found previously that a β-globin cDNA linked to the β-globin promoter in the microlocus cassette was not expressed at high levels in MEL cells unless additional β-globin gene sequences were included (Collis *et al.*, 1990), we tested the effect of adding further β-globin gene sequences to the *lacZ* reporter gene. Three different constructs were tested. In βC (Figure 1), a DNA fragment from the *Bam*HI site in the second exon of the β-globin gene to the *Eco*RV site located 1.7 kb past the polyadenylation signals of the gene was cloned after the SV40 polyadenylation signals in the βB construct. This β-globin fragment includes the second intron, third exon, polyadenylation signals and the downstream enhancer that was

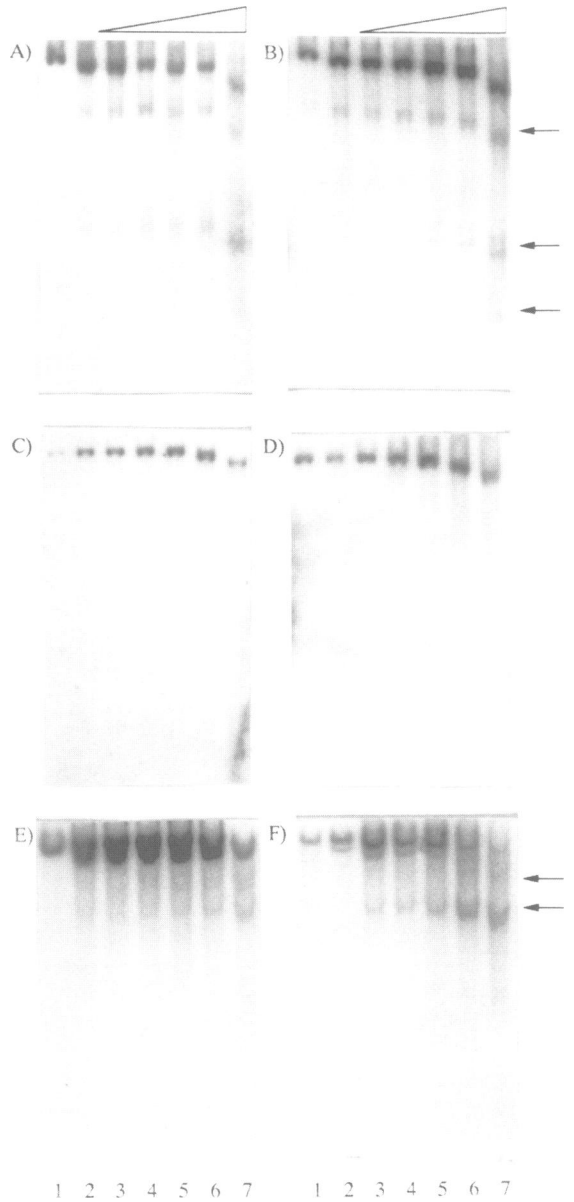

Fig. 4. Hypersensitive site determination. Nuclei isolated from 15.5 day fetal livers were assayed for DNase I hypersensitivity of the transgene LCR or the endogenous mouse β-globin LCR. (**A–D**) Transgenic lines βB5, βB6, βB1 and βB7, respectively, were analysed with a human HSS1 probe from the transgene LCR. βB1 and βB7 were also analysed with a mouse LCR probe in (**E**) and (**F**), respectively. In lanes 1, DNA was isolated from untreated fetal liver nuclei, while in lanes 2–7 the DNA was isolated from nuclei that were incubated with increasing amounts of DNase I (0, 0.1, 0.4, 1.0, 4.0 and 16 μg, respectively). The arrows to the right of the figure indicate the position of DNA fragments that appear on the Southern blot due to the presence of DNase I-hypersensitive sites in the respective LCR.

mapped previously (Behringer *et al.*, 1987; Kollias *et al.*, 1987; Trudel and Costantini, 1987). However, since the SV40 polyadenylation signals will be used in this construct, the downstream β-globin gene will not be part of the unprocessed mRNA. In βD (Figure 1) the same β-globin gene fragment was cloned 3′ of *lacZ* in the βB construct, but the SV40 polyadenylation signals were removed in this case so that the β-globin sequences will become part of the transcription unit. The second intron will be spliced out and the β-globin gene polyadenylation

Table II. Analysis of βC, βD and βE transgenic fetuses

Line[a]	β-gal. (mU/mg)	Copy no.[b]	Expression/copy (mU/mg)/copy
βC1 (l)	1650	20	82
βC2 (l)	1020	10	100
βC3 (l)	105	16	6.6
βD1	2750	50	55
βD2	125	3	42
βD3	15	5	3.0
βD4	30	12	2.5
βD5	750	5	150
βD6	3	1	3.0
βD7	130	3	43
βD8	1060	12	88
βD9	3900	25	156
βD10	300	15	20
βD11	300	2	150
βD12	2	1	2.0
βD13	5	30	0.2
βD14	50	20	2.5
βD15 (l)	12	20	0.6
βD16 (l)	23	30	0.8
βD17 (l)	1020	14	73
βE1	10	5	2.0
βE2	6	4	1.5
βE3	9	0.6	15
βE4	6	4	1.5
βE5	1	2	0.5
βE6	120	2	60
βE7	8	1	8.0
βE8	50	1	50
βE9	1	5	0.2
βE10	25	0.7	36

[a](l) depicts bred lines which were analysed as in Table I. The other mice were analysed as founder fetuses 15 days following microinjection.
[b]The copy number was determined using fetal liver DNA.

signals will be used. Although a hybrid mRNA will be produced, the β-galactosidase protein synthesized will be the same because the *lacZ* translational stop site is present. In the third construct (βE), the *lacZ* gene, without a promoter or SV40 polyadenylation signals, was cloned directly into the *Nco*I site situated at the protein initiation codon (ATG) in exon 1 of the β-globin gene. Thus, the whole β-globin gene is present in this case. Introns 1 and 2 will be spliced from the precursor message and the β-globin polyadenylation signals will be used. For these three constructs, fetal livers of founder embryos were studied directly in most cases, although a few lines were also bred. When founder fetal livers were tested directly it is possible that some animals were mosaic. However, this should not have affected the results as the transgene copy number was determined using fetal liver DNA in these cases; the same tissue used to measure β-galactosidase activity. If the fetal liver was mosaic for the transgene, expression and copy number would be affected in the same manner. All transgenes were also tested for deletions as described above. None were detected in transgenic animals that are included in the analyses.

Three lines carrying the βC construct were established and between them there was a 15-fold variation in expression per transgene copy (compare βC2 and βC3 in Table II). Many more lines and/or founders carrying the βD or βE transgenes were generated and the level of β-galactosidase expression per copy was seen to vary enormously for both

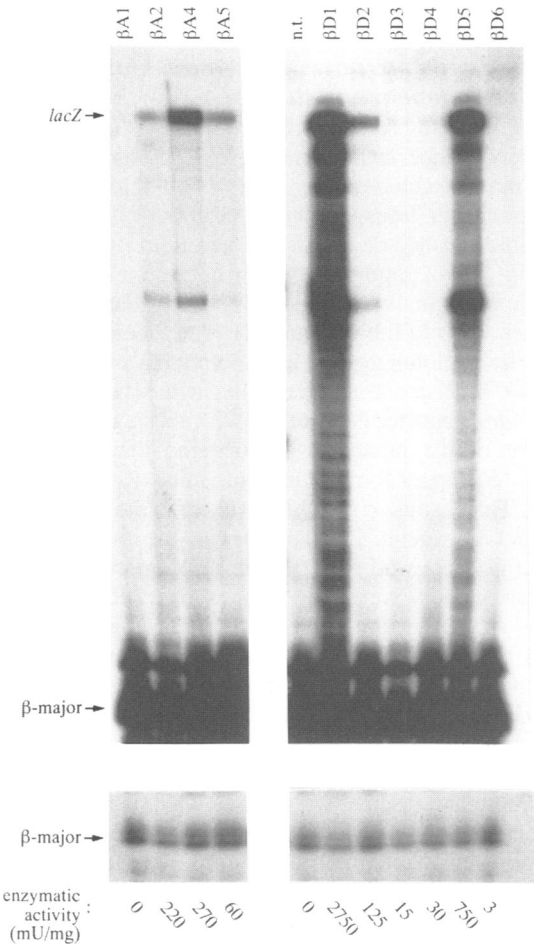

Fig. 5. RNase protection analysis of *lacZ* mRNA expression. Total RNA from the fetal liver of the βA line or βD founder indicated at the top of each lane (n.t., non-transgenic) was assayed by RNase protection using *lacZ* and β-major antisense probes. The position of the signals for *lacZ* and β-major are indicated by the arrows to the left of the figure. The top panel shows a 1 week autoradiographic exposure of the complete gel, while in the bottom panel a 5 h exposure of the β-major signal is also shown for clarity. For comparison, the level of β-galactosidase activity measured in fetal liver extracts of each transgenic line or founder is indicated at the bottom of each lane.

of these constructs (Table II). For βD, a >700-fold variation was observed (compare βD9 and βD13), while for βE a 300-fold variation was obtained (compare βE6 and βE9). Moreover, several of the βD and βE transgenic founders (βD6, βD12, βE5 and βE9 in Table II) gave total levels of activity that were near or only a few fold above the background, which was determined to be 1 mU/mg. Thus, although all the transgenic derivatives with the βD and βE constructs demonstrated detectable levels of expression, in addition to showing a high variation in the level of expression per transgene copy, the absolute level of expression in some cases was extremely low.

β-Galactosidase activity levels in extracts correspond to lacZ mRNA levels

To test whether the levels of β-galactosidase activity in extracts reflected the levels of transgene message, fetal liver RNA from several βA lines and βD founders was assayed by RNase protection for *lacZ* mRNA expression and for mouse β-major globin expression as an internal control (Figure 5). The β-major signal varied only slightly among the different samples. In contrast, the *lacZ* signal varied tremendously, and this variation correlated with the amount of β-galactosidase activity measured in extracts, which is given at the bottom of the lanes in Figure 5 for comparison. Thus, the β-galactosidase activity accurately reflected the amount of *lacZ* mRNA produced in the different transgenic animals.

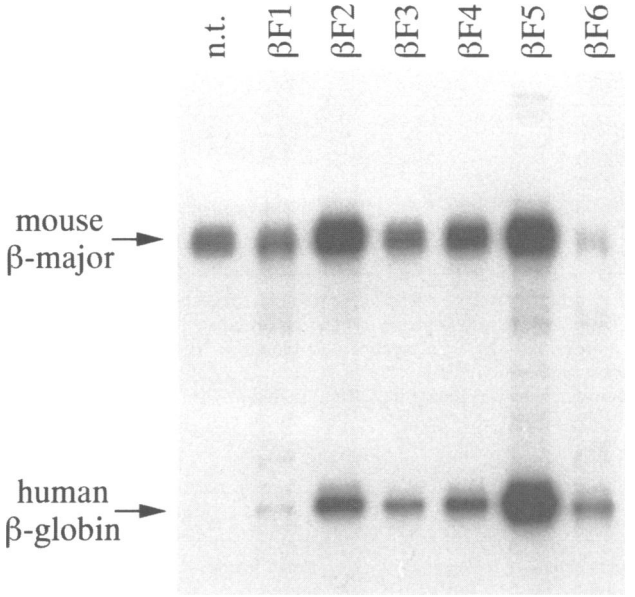

Fig. 6. S1 nuclease protection analysis of β-globin gene expression in mice carrying the β-globin gene without *lacZ* sequences. Total RNA, isolated from the fetal liver of the founder transgenic mouse indicated at the top of each lane (n.t., non-transgenic) carrying the βF construct, was assayed by S1 protection for human β-globin mRNA and for the endogenous mouse β-major transcript.

The LCR does confer position-independent expression onto the β-globin gene itself

The microlocus LCR does confer position-independent, copy number-dependent expression onto the β-globin gene itself in transgenic mice (Talbot *et al.*, 1989). However, the version of the microlocus used in that study was slightly different from that designed by Collis *et al.* (1990). To ensure that the microlocus LCR used in the present study could confer position-independent expression onto the β-globin gene alone and to determine what variation in expression per copy would be obtained, the human β-globin gene alone in the same microlocus was studied (βF construct in Figure 1). Founder transgenic fetal livers were tested for expression of the human β-globin transgene mRNA relative to the endogenous mouse β-major mRNA by S1 protection analysis (Figure 6). Six out of six independent founders showed high level expression of the transgene and there was <4-fold variation in expression per transgene copy (summarized in Table III). Thus, this version of the LCR does confer position-independent expression onto the β-globin gene.

In drug-resistant-selected MEL cell clones, a LCR–lacZ transgene shows a much closer relationship between copy number and expression levels

For comparative purposes, the βD construct, for which we had obtained the most independent transgenic samples

Table III. Analysis of βF transgenic fetuses[a]

Line	% huβ/moβ[b]	Copy no.[c]	Expression/copy
βF1	11	0.2	55
βF2	37	1	37
βF3	50	3	17
βF4	75	5	15
βF5	180	8	23
βF6	328	6	55

[a]Fetal livers were isolated 14 days after microinjection.
[b]The ratio of human β-globin expression to mouse β-major expression was determined by S1 protection (see Figure 5). The copy number was determined as in Table II.
[c]Copy number was determined from the fetal liver DNA.

Table IV. Analysis of MEL clones transfected with βD construct

Transfected clone	β-gal. (mU/mg)	Relative copy no.[a]	Expression/copy
βD-C1	570	2	290
βD-C2	1200	2	600
βD-C3	1200	2	600
βD-C4	1300	2	650
βD-C5	2300	4	575
βD-C6	1600	6	270
βD-C7	1300	3	430
βD-C8	3300	4	830
βD-C9	800	2	400
βD-C10	1100	2	550
βD-C11	510	3	170
βD-C12	1000	1	1000
βD-C13	970	2	490
βD-C14	1300	1	1300
βD-C15	780	2	390
βD-C16	710	2	355
βD-C17	620	2	310
βD-C18	630	6	105
βD-C19	510	2	260
βD-C20	410	1	410
βD-C21	270	1	270
βD-C22	430	1	430
βD-C23	360	2	180

[a]A copy number of 1 was assigned to the clone with the lowest LCR probe intensity relative to the *dt* control probe intensity on Southern blot analysis and the others were assigned by comparison with it.

(Table II), was also transfected into MEL cells. In this case, the vector contained the G418 drug-selectable gene *neo* that was removed in preparing DNA for the generation of transgenic mice. Individual, stably transfected clones were isolated and DNA was analysed by Southern blot to determine transgene copy number and to test for deletions. Clones that showed no deletions were induced to differentiate with dimethylsulfoxide, and extracts from the cells were measured for β-galactosidase activity (Table IV). All of the clones gave relatively high levels of enzymatic activity. Moreover, the levels of expression per transgene copy correlated to a much better degree than the same construct studied in transgenic mice. There was <13-fold variation in expression on a per transgene copy basis in MEL clones versus a >700-fold variation for the same construct studied in transgenic mice (compare results for βD construct in Table IV with those in Table II).

Discussion

Cultured cells are not a good model for position-independent studies

Although the microlocus LCR confers position-independent, copy number-dependent expression onto the β-globin gene in transgenic mice, with a <4-fold variation in expression per transgene copy (Talbot *et al.*, 1989, and see Table III), we demonstrated here that LCR constructs running a *lacZ* reporter gene in mice give up to a 700-fold variation in expression of β-galactosidase activity per transgene copy (Tables I and II). This occurs even when the entire β-globin gene is incorporated as part of the *lacZ* reporter gene construct. Recently, it has been suggested that a *lacZ* reporter gene *in cis* with the major regulatory element of the human α-globin gene locus gives cell-variegated expression in transgenic mice (Robertson *et al.*, 1995). This results in variable levels of expression between different transgenic lines. However, the β-globin LCR–*lacZ* constructs studied in the present report do not show cell-variegated expression (manuscript submitted). Our results show that the β-globin LCR is unable to confer position-independent, copy number-dependent expression onto the *lacZ* reporter gene in transgenic mice.

In contrast to the results in transgenic mice, an LCR–*lacZ* construct gave only 13-fold variation in expression per copy in stably transfected clones of MEL cells (Table IV). There are at least two reasons why such a difference between the mouse and cell culture models may exist. (i) Stably transfected cell clones are drug selected. For a clone to be able to grow in the presence of drug, the promoter of the selectable gene must be at least mildly active. If it happened that the LCR were unable to open chromatin itself, one would then only select clones in which the transgene had integrated into sites of chromatin that are normally transcriptionally active. In such cases, the LCR enhancers may be able to function at high levels independently from the LCR chromatin opening activity. On the other hand, both transcriptionally inactive and active sites of chromatin integration are obtained in transgenic mice, as no selection for the transgene is used. Thus, transgene expression would be expected to show position effects in mice if the LCR were unable to open chromatin properly. (ii) Alternatively, the LCR in our constructs may be more able to open chromatin properly and allow the LCR enhancers to function in a position-independent manner in MEL cells than in transgenic mice. During development, chromatin remodelling occurs at different stages and acts to activate and repress different regions of chromatin in a tissue-specific manner (Boulikas, 1991; Zlatanova and Holde, 1992). For the LCR to function fully in erythroid cells and in any chromatin site in the developing organism it must be able to override the mechanisms that are attempting to repress gene expression at each developmental stage. On the other hand, MEL cells represent a single and late (adult) stage of mouse development. The requirements for the LCR to open and maintain chromatin in an active configuration may therefore be less stringent in MEL cells than in transgenic mice. Whatever the reason, our results unequivocally show that an LCR-containing construct that does not give position-independent expression in transgenic mice can give a false indication of position independence in cell

culture clones. Thus, drug-selected cell culture clones are not a good model for studies regarding position-independent expression. Others have also demonstrated that culture cells may sometimes be a poor model for gene expression studies. For example, Zimmerman *et al.* (1990) found that expression of N-*myc* transgenes was tissue restricted in mice but not in culture cells.

Why does the LCR not confer position-independent expression onto the lacZ reporter gene in transgenic mice?

Previously, when globin genes were studied without the LCR, it was found that the β-globin promoter alone, as the only defined regulatory sequence, was not sufficient for expression in transgenic mice (Behringer *et al.*, 1987; Trudel and Costantini, 1987). However, with the β-globin promoter extending to –800 linked to the *lacZ* gene (βB construct), or with the promoter extending to just –87 (βA construct), with no additional regulatory elements other than the LCR, seven of seven and three of five transgenic lines, respectively, gave detectable β-galactosidase activity in mice (Table I). Thus, the enhancers of the LCR can work at least partly in transgenic mice with constructs containing the *lacZ* gene. Moreover, the *lacZ* reporter gene was expressed at high levels in every MEL cell clone and in a near copy number-dependent fashion (Table IV). Thus, in the context of active chromatin, regardless of whether or not it was the LCR that provided the active chromatin in MEL cells, the LCR enhancers are capable of invoking high levels of transcription onto the *lacZ* reporter gene. However, *lacZ* expression in transgenic mice was influenced greatly by position, suggesting that, in the presence of the *lacZ* reporter gene, the chromatin opening activity of the LCR is definitely impaired.

There may be many reasons why *lacZ* is able to interfere with the chromatin opening activity of the LCR in mice but still allow the enhancers to partly function. Two possibilities, that are dealt with individually below, are suggested by previous findings. The first possibility is that the LCR chromatin opening activity and its enhancer properties are partially or entirely separable functions that might involve different mechanisms. If this were the case, the *lacZ* sequences might be able to interfere with the chromatin opening function without affecting enhancer activity directly. That the two LCR properties are separable entities is suggested by the finding that the element of the chicken globin locus that can confer position-independent expression has only weak enhancer activity (Reitman *et al.*, 1990), and by the recent demonstration by one of us (Ellis *et al.*, 1996) that the chromatin opening activity of the human β-globin LCR, as measured by the ability to give position-independent expression in mice containing single copy transgenes, resides solely in the HSS3 region. Thus, the chromatin opening activity in the human β-globin LCR is both physically and functionally separate from the enhancers in HSS2 and HSS4. It remains to be determined if it is also separable from the enhancer activity of HSS3.

Do the chromatin opening activity and enhancer properties of the LCR involve different mechanisms? To explain why the globin genes compete for activation by the LCR during development, it has been suggested that the LCR functions through a looping mechanism, in which it interacts directly with the gene it is activating (Hanscombe *et al.*, 1991). This model has been supported strongly by further experimentation (Kim *et al.*, 1992; Fiering *et al.*, 1995). However, in most investigations, only the relative level of gene expression is considered. As the levels of transcription only reflect enhancer activity *per se*, the studies have not addressed directly whether the chromatin opening activity may function through a similar mechanism to the enhancers. In fact, the demonstration that a γ- and β-globin gene together *in cis* with the LCR are both expressed in a position-independent manner at all stages of development, although the genes do show a developmental-specific competition for levels of expression (Li and Stamatoyannopoulos, 1994), suggests that the genes compete for the enhancer activity but not for the chromatin opening activity of the LCR. This would be explained if LCR chromatin opening activity involves a mechanism different from that of the enhancers. As an example of an alternative to the looping model, a scanning mechanism has been suggested for other systems (Migeon, 1994; Hecht *et al.*, 1995). In a scanning scenario, a change in chromatin structure may originate and propagate from the LCR. There may exist sequences in the *lacZ* gene that block the necessary alteration in chromatin from passing through them, but do not directly prevent the LCR enhancers from interacting with the β-globin promoter. This would result in the position-dependent expression in transgenic mice we observe with LCR–*lacZ* constructs.

The second plausible explanation as to why the β-globin LCR is unable to activate the *lacZ* reporter gene in a position-independent manner is that the LCR chromatin opening activity is spatially distributed and requires a specific arrangement of defined regulatory sequences. For example, although the chicken β-globin gene *in cis* with its downstream enhancer (LCR) is expressed independently from position in mice, addition of any one of four upstream HSS regions, but not all four together, causes the gene to be transcribed in a position-dependent manner (Reitman *et al.*, 1995). In addition, whereas a human γ-globin gene with its promoter to –382, *in cis* with the LCR, is expressed independently of position, extension of the promoter to –730 prevents it, while also including the β-globin gene in 3′ permits the recovery of position-independent expression (Li and Stamatoyannopoulos, 1994). Thus, LCR chromatin opening activity may need specific regulatory sequences at both the LCR and gene level, as well as a specific spatial arrangement of these sequences. Although the β-globin gene would be expected to provide the necessary gene elements, as it is activated in a position-independent fashion by the LCR, inserting the *lacZ* gene into the β-globin gene might disturb the spatial arrangement required and/or prevent interactions between the different elements.

The two possibilities discussed above are not mutually exclusive. The LCR chromatin opening activity may involve a different mechanism from that of the enhancer activity and at the same time need a specific arrangement of regulatory sequences to function properly. In any case, our results have shown that the LCR does not confer position-independent expression onto just any gene in transgenic mice. Future work needs to be focused on determining what is required at the gene level for the LCR to be able to confer position-independent expression and/or whether chromatin opening functions as a separate

entity from enhancement. The *lacZ* gene may be a useful tool in this regard.

Materials and methods

Transgenic mouse production

Microlocus constructs were digested with *Sac*II to remove plasmid and *neo* gene sequences, the DNA fragments generated were separated by agarose gel electrophoresis, and the fragment of interest was purified using GeneClean II (Bio/Can Scientific). Transgenic mice were produced as described (Bérard *et al.*, 1994). Transgenic founders were either allowed to go to term and bred to produce stable lines or sacrificed 14 or 15 days following microinjection.

DNA analysis

For bred lines, tail DNA was used to determine copy number and transgene integrity. Fetal liver DNA was used for non-bred founders. The βA5 line was deemed to contain a single transgene copy by end fragment analysis (not shown), and was used as a standard to determine copy number for all other founders and lines. Fifteen μg of genomic DNA was digested with *Sac*I and analysed by Southern blot, hybridizing successively to a 2 kb *Xba*I fragment from the 5′ end of the LCR in the microlocus vector and a 1.3 kb *Sac*I fragment from the *dystonia musculorum* (*dt*) locus (Brown *et al.*, 1995) as a loading control. The signals were quantified by scanning autoradiograms with an LKB Ultra Scan laser densitometer linked to an LKB 2200 integrator.

β-Galactosidase assays

Transgenic fetuses were isolated 15 days after observation of the spermatic plug for bred fetuses or 15 days after microinjection for founder fetuses. For *in situ* detection of β-galactosidase activity, fetuses were fixed for 1 h in the dark at 4°C in 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40 and 1 mM $MgCl_2$ in phosphate-buffered saline (PBS). The fetuses were rinsed twice in PBS and then incubated for the indicated time in the dark at 37°C in PBS containing 0.01 M potassium ferrocyanide, 0.01 M potassium ferricyanide, 0.1 M 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) and 1 mM $MgCl_2$. For adult blood cells, a few drops of tail blood were collected, the cells were fixed in 0.2% glutaraldehyde in PBS for 10 min, rinsed in PBS and incubated for 18 h in X-gal solution as above. MEL cells were assayed *in situ* as described for adult blood.

To measure β-galactosidase activity in extracts, a quarter of a fetal liver was pipetted through a plastic pipette tip to disperse the cells into 100 μl of 0.25 M Tris–HCl (pH 8). Similarly, 1×10^5 induced MEL cells were diluted in 100 μl of the same buffer. The suspensions were frozen on dry ice and thawed at 37°C three times, and the insoluble matter was removed by centrifugation for 5 min at 4°C. Protein concentration was estimated using the Bio-Rad Protein Assay system (Bio-Rad Laboratories) with bovine serum albumin as a standard. Twenty μg of extract protein was assayed for β-galactosidase activity as described (Sambrook *et al.*, 1989) and activity was measured relative to a commercial β-galactosidase standard (Boehringer Mannheim). All assays were performed under linear conditions and several concentrations of the standard were assayed for different times in each case.

Hypersensitive site detection

DNase I-hypersensitive sites were measured as described (Forrester *et al.*, 1990) with modifications. Three to five fetal livers were homogenized in 5 ml of 10 mM Tris–HCl (pH 7.5) containing 10 mM NaCl and 0.1% NP-40 on ice and 20 ml of the same buffer without NP-40 was added. Nuclei were pelleted and resuspended into 1.6 ml of 10 mM Tris–HCl (pH 7.5) containing 10 mM NaCl and 1 mM $CaCl_2$. Aliquots were incubated for 5 min at 37°C with 0, 0.1, 0.4, 1, 4 or 16 μg of DNase I (Boehringer Mannheim). Genomic DNA was isolated and digested with *Cla*I and *Xho*I, and Southern blots of the DNA were hybridized with a 1 kb *Not*I–*Cla*I fragment (HSS1) from the human LCR in the microlocus vector. Alternatively, the DNA was digested with *Pst*I and Southern blots were hybridized with a 1.1 kb *Eco*RI–*Pst*I fragment from the mouse LCR (Jimenez *et al.*, 1992).

RNA analysis

The βF founder fetal livers (Table III) were isolated at 14 days following microinjection. Cells from half of a liver were dispersed in 500 μl of Trizol (Gibco-BRL) and RNA was isolated as described by the manufacturer. DNA from the cell debris in the organic phase was precipitated with 1 ml of ethanol and purified by usual procedures for tissues. S1 analysis was done as described (Ellis *et al.*, 1993). The βA and βD fetal livers (Figure 5) were isolated at 15.5 days and RNA purified as above. RNase protection assays were performed as described previously (Delvoye *et al.*, 1993). The antisense RNA probes used represented a *lacZ* gene fragment from *Cla*I to *Eco*RV (+1129 to +1417 relative to the translation initiation site) in the *lacZ* gene that protects a 288 base RNA fragment and a β-major fragment from *Sau*3A to *Hga*I (–15 to +60 relative to the Cap site) of the mouse β-major gene that protects a 60 base RNA fragment.

Cell culture

MEL (C88) cells were grown and transfected, as previously described (Delvoye *et al.*, 1993). Single clones were selected by serially diluting cell suspensions to contain on average one cell per 200 μl in 20% conditioned medium, plating 200 μl in individual wells of 96-well plates and microscopically testing for individual cells and colonies for several days. Selected clones were induced with 2% dimethylsulfoxide for 5 days and protein extracts for β-galactosidase measurements were prepared.

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References

Behringer,R.R., Hammer,R.E., Brinster,R.L., Palmiter,R.D. and Townes, T.M. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 7056–7060.

Bérard,J., Gaboury,L., Landers,M., De Repentigny,Y., Houle,B., Kothary,R. and Bradley,W.E. (1994) *EMBO J.*, **13**, 5570–5580.

Blom van Assendelft,G., Hanscombe,O., Grosveld,F. and Greaves,D.R. (1989) *Cell*, **56**, 969–977.

Bonifer,C., Yannoutsos,N., Kruger,G., Grosveld,F. and Sippel,A.E. (1994) *Nucleic Acids Res.*, **22**, 4202–4210.

Boulikas,T. (1991) *Anticancer Res.*, **11**, 489–527.

Brown,A., Bernier,G., Mathieu,M., Rossant,J. and Kothary,R. (1995) *Nature Genet.*, **10**, 301–306.

Chada,K., Magram,J. and Costantini,F. (1985) *Nature*, **319**, 685–689.

Collis,P., Antoniou,M. and Grosveld,F. (1990) *EMBO J.*, **9**, 233–240.

Costantini,F., Radice,G., Magram,J., Stamatoyannopoulos,G., Papayannopoulou,T. and Chada,K. (1985) *Cold Spring Harbor Symp. Quant. Biol.*, **50**, 361–370.

Delvoye,N.L., Destroismaisons,N.M. and Wall,L.A. (1993) *Mol. Cell. Biol.*, **13**, 6969–6983.

Dillon,N. and Grosveld,F. (1993) *Trends Genet.*, **9**, 134–137.

Ellis,J., Talbot,D., Dillon,N. and Grosveld,F. (1993) *EMBO J.*, **12**, 127–134.

Ellis,J., Talbot,D., Tan-Un,K.C., Harper,A., Michalocich,D., Yannoutsos,N., Philipsen,S. and Grosveld,F. (1996) *EMBO J.*, **15**, 562–568.

Epner,E., Kim,C.G. and Groudine,M. (1992) *Curr. Biol.*, **2**, 262–264.

Fiering,S., Epner,E., Robinson,K., Zhuang,Y., Telling,A., Hu,M., Martin,D.I.K., Enver,T., Ley,T.T. and Groudine,M. (1995) *Genes Dev.*, **9**, 2203–2213.

Forrester,W.C., Thompson,C., Elder,J.T. and Groudine,M. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 1359–1363.

Forrester,W.C., Epner,E., Driscoll,M.C., Enver,T., Brice,M., Papayannopoulou,T. and Groudine,M. (1990) *Genes Dev.*, **4**, 1637–1649.

Greaves,D.R., Wilson,F.D., Lang,G. and Kioussis,D. (1989) *Cell*, **56**, 979–986.

Grosveld,F., van Assendelft,G.B., Greaves,D.R. and Kollias,G. (1987) *Cell*, **51**, 975–985.

Hanscombe,O., Whyatt,D., Fraser,P., Yannoutsos,N., Greaves,D., Dillon,N. and Grosveld,F. (1991) *Genes Dev.*, **5**, 1387–1394.

Hecht,A., Laroche,T., Strahl-Bolsinger,S., Gasser,S.M. and Grunstein,M. (1995) *Cell*, **80**, 583–592.

Huber,M.C., Bosch,F.X., Sippel,A.E. and Bonifer,C. (1994) *Nucleic Acids Res.*, **22**, 4195–4210.

Jimenez,G., Griffiths,S.D., Ford,A.M., Greaves,M.F. and Enver,T. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 10618–10622.

Kim,C.G., Epner,E.M., Forrester,W.C. and Groudine,M. (1992) *Genes Dev.*, **6**, 928–938.

Kollias,G., Hurst,J., deBoer,E. and Grosveld,F. (1987) *Nucleic Acids Res.*, **15**, 5739–5747.
Li,Q. and Stamatoyannopoulos,J.A. (1994) *Mol. Cell. Biol.*, **14**, 6087–6096.
Madisen,L. *et al.* (1994) *Genes Dev.*, **8**, 2212–2226.
May,G. and Enver,T. (1995) *EMBO J.*, **14**, 564–574.
Migeon,B.R. (1994) *Trends Genet.*, **10**, 230–235.
Palmiter,R.D., Sandgren,E.P., Koeller,D.M. and Brinster,R.L. (1993) *Mol. Cell. Biol.*, **13**, 5266–5275.
Philipsen,S., Talbot,D., Fraser,P. and Grosveld,F. (1990) *EMBO J.*, **9**, 2159–2167.
Pruzina,S., Hanscombe,O., Whyatt,D., Grosveld,F. and Philipsen,S. (1991) *Nucleic Acids Res.*, **19**, 1413–1419.
Reitman,M., Lee,E., Westphal,H. and Felsenfeld,G. (1990) *Nature*, **348**, 749–752.
Reitman,M., Lee,E., Westphal,H. and Felsenfeld,G. (1993) *Mol. Cell. Biol.*, **13**, 3990–3998.
Reitman,M., Lee,E. and Westphal,H. (1995) *Nucleic Acids Res.*, **23**, 1790–1794.
Robertson,G., Garrick,D., Wu,W., Kearns,M., Martin,D. and Whitelaw,E. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 5371–5375.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Stamatoyannopoulos,G. and Nienhuis,A.W. (1994) Hemoglobin switching. In Stamatoyannopoulos,G., Nienhuis,A.W., Majerus,P.W. and Varmus,H. (eds), *The Molecular Basis of Blood Diseases*. W.B Saunders Co., Philadelphia, PA, pp. 107–136.
Stamatoyannopoulos,J.A., Goodwin,A., Joyce,T. and Lowrey,C.H. (1995) *EMBO J.*, **14**, 106–116.
Talbot,D., Collis,P., Antoniou,M., Vidal,M., Grosveld,F. and Greaves, D.R. (1989) *Nature*, **338**, 352–355.
Talbot,D., Philipsen,S., Fraser,P. and Grosveld,F. (1990) *EMBO J.*, **9**, 2169–2177.
Talbot,D., Descombes,P. and Schibler,U. (1994) *Nucleic Acids Res.*, **22**, 556–566.
Townes,T.M. and Behringer,R.R. (1990) *Trends Genet.*, **6**, 219–223.
Townes,T.M., Lingrel,J.B., Chen,H.Y., Brinster,R.L. and Palmiter,R.D. (1985) *EMBO J.*, **4**, 1715–1723.
Trudel,M. and Costantini,F. (1987) *Genes Dev.*, **1**, 954–961.
Tuan,D., Solomon,W., Li,Q. and London,I.M. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 6384–6388.
Zimmerman,K., Legouy,E., Stewart,V., Depinho,R. and Alt,F.W. (1990) *Mol. Cell. Biol.*, **10**, 2096–2103.
Zlatanova,F.S. and Holde,K.E. (1992) *Crit. Rev. Eucaryot. Gene Expr.*, **2**, 211–224.

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