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Molecular analysis of the human β-globin locus activation region

(globin genes/chromatin/DNase I hypersensitive sites/erythroid specific)

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ABSTRACT **Recently, DNA sequences containing four erythroid-specific DNase I hypersensitive sites within 20 kilobases 5′ of the human ε-globin gene have been identified as an important cis-acting regulatory element, the locus activation region (LAR). Subfragments of the LAR, containing either all or only the two 5′ or two 3′ hypersensitive sites were linked to the human β-globin gene and analyzed for their effect on globin gene expression in stably transformed mouse erythroleukemia (MEL) cells. Constructs containing all four of the hypersensitive sites increase β-globin mRNA levels 8- to 13-fold, while constructs with only the 5′ or 3′ sites increase globin expression to a lesser extent. No effect was seen when the constructs were assayed in 3T3 fibroblasts. All of the LAR derivatives form hypersensitive sites at the corresponding sequence position in MEL cells prior to and after induction of MEL cell differentiation. However, in 3T3 fibroblasts only the hypersensitive site corresponding to the previously described erythroid-specific −10.9 site was formed.**

The human β-like globin gene locus consists of six linked genes in the 5′ to 3′ order ε-γ^G-γ^A-$\psi\beta$-δ-β spanning ≈50 kilobases (kb) of DNA (reviewed in ref. 1). In human fetal liver, adult erythroid cells, and erythroleukemic cell lines, different globin genes are transcribed, yet the entire β-like globin gene locus is preferentially sensitive to DNase I digestion (2, 3). In addition, two classes of DNase I hypersensitive sites have been described in the β-globin locus. The first are erythroid and developmentally specific and are located at the 5′ ends of the globin genes, which are transcriptionally active in a particular tissue or cell line (2). A second class of hypersensitive sites located 6–18 kb 5′ to the ε- and 20 kb 3′ to the β-globin genes are erythroid specific and developmentally stable and are present regardless of which globin genes are active (3, 4).

Several observations suggest that the developmentally stable hypersensitive sites are important in the activation of the β-globin locus. In a specific subset of the $(\gamma\delta\beta)^0$-thalassemia class of mutations, deletions of the region containing the hypersensitive sites 5′ to the ε-globin gene result in the failure to transcriptionally activate the remaining globin genes and leave the affected locus in an inactive DNase I-resistant chromatin structure (5, 6). In addition, transactivation of the human adult β-globin gene in somatic cell hybrids resulting from the fusion of human nonerythroid cells and murine erythroleukemia (MEL) cells (7) is accompanied by a reorganization of ≈80 kb of chromatin into a DNase I-sensitive domain, including formation of the four developmentally stable hypersensitive sites 50 kb 5′ to the activated adult β-globin gene (8). Thus, the region containing these developmentally stable hypersensitive sites was termed "locus activation region" or LAR (8). Direct support for this model was obtained by the demonstration that inclusion of ≈40 kb of sequence containing the 5′ and 3′ LARs in β-globin constructs introduced into transgenic mice resulted in high-level copy number-dependent and position-independent expression of the β-globin gene (9). This is in marked contrast to the low level, variable, position-dependent and copy number-independent expression observed when the β-globin gene lacking the LAR is introduced into mice (10, 11) or MEL cells (12).

To further understand the relationship between the LAR and the high-level expression of the linked β-globin gene, we have constructed cassettes containing DNA sequences corresponding to all four or subsets of the 5′ developmentally stable hypersensitive sites. Our results suggest that as little as 2.5 kb containing DNA sequences corresponding to the four 5′ developmentally stable hypersensitive sites is sufficient to confer high-level expression of a linked β-globin gene in MEL cells. In addition, the β-globin gene linked to cassettes containing two of the four hypersensitive sites is expressed at approximately half the level conferred by the cassette containing all four hypersensitive site sequences. In all cases, the LAR-associated hypersensitive sites were formed after transfection of the cassettes into MEL cells, both prior to and after induction.

MATERIALS AND METHODS

Plasmid Constructs. The DNA sequence of the 5′ ε region is available in GenBank (accession no. J00179). In describing the individual constructs, restriction sites lost during cloning are denoted with an asterisk (*); numbers in parentheses refer to distance in kb relative to the major ε-globin transcription initiation site, and other numbering is from the GenBank file. pminiLAR-β (Fig. 1) includes the following sequences, which correspond to the developmentally stable hypersensitive sites 6.1, 10.9, 14.7, and 18 kb 5′ to the ε-globin gene: *Eco*RI at 17.482 (−2.061) to *Bgl* II 16.908 (−2.250)*; *Eco*RV 15.180 (−4.360)* to *Eco*RI 10.947 (−8.594)*; *Bgl* II 9.218 (−10.322)* to *Kpn* 7.764 (−11.776)*; *Pvu* II 5.122 (−14.418)* to *Sac* 4.274 (−15.263); *Sac* 2.199 (−17.341) to *Sac* 0.951 (−18.589). Derivatives of the mini(m)LAR were made as follows (sizes in parentheses indicate amount of LAR sequences in kb): m6,11β (5.7), deletion of sequences between *Bam*HI (position 5.9) and *Cla* (position 8.0) from mLARβ; m15,18β (2.3), deletion of sequences between *Sma* (position 1.5) and *Sma* (position 5.5) from mLARβ; micro(μ)LARβ (2.5 kb) was not derived from mLARβ but from subclones that contain the individual hypersensitive sites; however, restriction fragments used are indicated in Fig. 1. This plasmid contains the following sequences: *Hin*dIII 13.769 (−5.771) to *Nco* I 13.062 (−6.478); *Bgl* II 9.218 (−10.322)* to *Hin*dIII 8.486 (−11.054); *Hin*dIII 5.172 (−14.368) to *Bal* I 4.608 (−14.932)*; *Sph* I 1.702 (−17.838)* to *Pst* 1.182 (−18.358). μ6,11β (1.5 kb) contains

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Abbreviations: MEL, mouse erythroleukemia; LAR, locus activation region.

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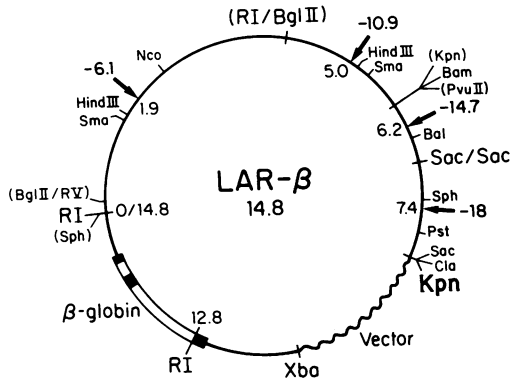

FIG. 1. Map of pmini-LAR-β (mLAR). Restriction sites are shown that demarcate fragments used to make the other LARβ derivatives. Restriction enzymes in parentheses indicate the loss of the enzyme recognition sequence as a result of cloning. Vector (pBluescript) sequences are shown as a wavy line. The human adult β-globin gene is shown as a boxed region; open box indicates introns, and solid box indicates exons.

the *Hin*dIII/*Nco* I and *Bgl* II/*Hin*dIII fragments described for the μLARβ above. m15,18β (1.0) contains the *Hin*dIII/*Bal* and *Sph*/*Pst* fragments described for the μLARβ.

The β-globin gene is contained on a 3.8-kb *Sph* I (0.613 kb 5′ to β) to *Xba* (1.7 kb 3′ to β) fragment. This β-globin gene fragment contains all previously described regulatory sequences within and 3′ to the gene, including the putative stage-specific enhancer and elements responsible for increased transcription after induction (13–16). All of the plasmids described above are propagated in pBluescript (Stratagene), which is ≈3 kb. For all plasmids used, the unique *Kpn* site in the pBluescript polylinker (highlighted in Fig. 1) was cleaved prior to electroporation.

Cell Culture and Electroporation. The semiadherent aprt⁻ MEL cell line (13) was grown in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and induced to differentiate with 2% dimethyl sulfoxide or 3 mM hexamethylenebisacetamide for 6 days. Electroporation was as described by Chu *et al.* (17) with 3 μg of pSV2*neo (ref. 18; linearized with *Eco*RI) and 10–20 μg of β-globin plasmid DNA (linearized with *Kpn*). The electric field was 500 V/cm, with 250 μF capacitance. Cells were plated into semisolid selective medium after 24 hr, and individual colonies were isolated and expanded for analysis. NIH 3T3 cell lines were obtained similarly.

Copy Number Determination. Total cellular DNA was isolated, digested with *Eco*RI, Southern blotted, and hybridized to an intronless human β-globin gene probe (19). This probe hybridizes to a 2.0-kb *Eco*RI fragment internal to all of the β-globin-containing constructs used in these experiments, as well as to junction fragments containing the 3′ end of the β-globin gene and mouse genomic sequences. The number of integrants was determined by counting the number of individual junction fragments as well as by examining the intensity of the 2.0-kb restriction fragment.

RNA Analysis. The ribonuclease protection assay, human and mouse β-globin probes, and quantitation of results are as described (19). The human β-globin probe protects fragments of 42 and 90 nucleotides (nt). Only the 90-nt human fragment is shown and quantified because of variable cross-hybridization between mouse globin RNA and the human globin probe producing protected fragments of 30–50 nt in control and electroporated MEL cell lines (19).

Chromatin Analysis. Methods are as described (2, 3, 8).

RESULTS

mLAR and μLAR Confer High-Level Expression. The first question we addressed was whether high-level expression of a human β-globin gene could be achieved in MEL cells by linkage to a LAR cassette from which the 3′ β hypersensitive site region and sequences between the four 5′ ε hypersensitive sites were deleted. In clones containing the β-globin gene without LAR sequences, the amount of human β-globin RNA per gene copy ranges from 3% to 16%, with an average of 10%, of the amount of mouse β^{major}-globin RNA in induced MEL cells (Fig. 2*A* and Table 1). This is in agreement with previous reports (12, 20). In contrast, all clones containing the human β-globin gene linked to the LAR or subsets of LAR sequences expressed high levels of human β-globin RNA after induction (Fig. 2). On a per gene basis, clones containing the mLAR/β-globin cassette contained 20–120% (average, 80%) the amount of human β-globin RNA compared to mouse β^{major}-globin RNA (Fig. 2*B* and Table 1). Similarly, clones

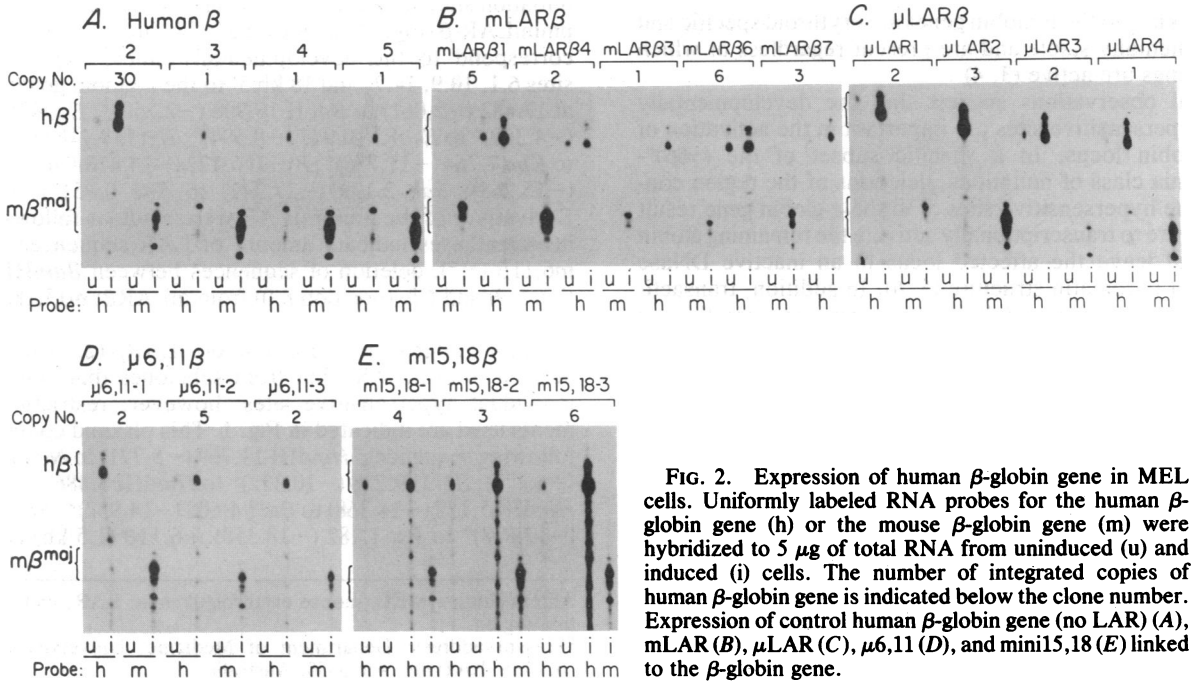

FIG. 2. Expression of human β-globin gene in MEL cells. Uniformly labeled RNA probes for the human β-globin gene (h) or the mouse β-globin gene (m) were hybridized to 5 μg of total RNA from uninduced (u) and induced (i) cells. The number of integrated copies of human β-globin gene is indicated below the clone number. Expression of control human β-globin gene (no LAR) (*A*), mLAR (*B*), μLAR (*C*), μ6,11 (*D*), and mini15,18 (*E*) linked to the β-globin gene.

Table 1. Quantitative comparison of human β-globin/mouse β-globin expression levels in MEL cells

Cell line	Copy	hβ RNA/ mβ^{maj} RNA
β1	1	0.1
β2	>30	<0.1
β3	1	0.03
β4	1	0.16
mLAR 1	5	0.5
mLAR 2	5	1.1
mLAR 3	1	1.1
mLAR 4	2	0.3
mLAR 5	1	1.2
mLAR 6	6	1.2
mLAR 7	3	0.2
μLAR 1	2	1.8
μLAR 2	2	0.5
μLAR 3	2	2.5
μLAR 4	1	0.6
μRAL 1	2	0.5
μRAL 2	2	2.2
m15,18 1	4	0.4
m15,18 2	3	0.4
m15,18 3	6	0.5
μ6,11 1	2	0.3
μ6,11 2	5	0.2
μ6,11 3	2	0.4

Cell lines, listed on left, which contain the human β-globin genes were analyzed for the number of integrated human β-globin genes by Southern blotting. Ratios of human β-globin RNA to mouse β^{major}-globin RNA (hβ RNA/mβ^{maj} RNA) in cells induced with 2% dimethyl sulfoxide were determined by counting the excised gel region corresponding to the protected globin mRNA fragment and correcting the amount of human β-globin RNA to the number of integrated genes. The ratio of induced human β-globin per human β-globin gene was normalized to the amount of endogenous mouse β^{major}-globin RNA in each clone, which is an internal control for the induced levels of globin RNA in these cells. The number of mouse globin genes in each of the aneuploid MEL clones is unknown; however, the ratio of the per copy expression of the human β-globin genes to mouse β^{major}-globin RNA provides a conservative estimate of the relative activity of the human to mouse genes since it is equivalent to expressing human to mouse globin gene activity, assuming one copy of the mouse globin per MEL cell.

into which the μLAR/β-globin constructs were introduced produced between 50% and 250% (average, 108%) the amount of human compared to mouse β^{major}-globin RNA, when normalized per human globin gene copy (Fig. 2*C* and Table 1). In two clones containing a cassette in which the μLAR was linked to the human β-globin gene in the reverse genomic orientation (μRAL), the amount of human β-globin RNA per gene was 50% and 220% (average, 135%) that of mouse β^{major}-globin RNA (Table 1). These results demonstrate that the mLAR and μLAR cassettes can increase the expression of a linked β-globin gene to levels that on average are similar to the expression of the endogenous mouse β-globin genes in MEL cells and that the effect of the LAR is orientation independent. The data also show clonal variation in the ratio of human to mouse β-globin expression. This could be due to the variable preinduced levels of human β-globin RNA observed in some LAR-β-containing cell lines (Fig. 2). In addition, variations in the extent of induction, the number of mouse β-globin genes, and/or alterations in the transfected LAR/β-globin gene cassettes may account for these clonal differences.

LAR Reductions. To further define the elements within the LAR responsible for the high-level expression of the linked β-globin gene, cassettes containing the human β-globin gene and LAR sequences corresponding to the −6 and −11 hypersensitive sites (simplified from −6.1 and −10.9) or the −15 (simplified from −14.7) and −18 sites were introduced into MEL cells. As shown in Table 1 and Fig. 2 *D* and *E*, human β-globin gene activity was severalfold higher in MEL clones containing these cassettes compared to MEL clones containing the β-globin gene alone. However, neither the 6,11 nor the 15,18 cassettes conferred as high expression as the complete mLAR or μLAR to the linked β-globin gene. In cells containing the mLAR 6,11 cassette, the levels of human β-globin RNA per gene copy ranged from 20% to 40% of mouse β^{major}-globin RNA (average, 30%), and in those containing the μLAR 15,18 cassette, the per gene copy expression of human β-globin gene was 40–80% that of mouse β^{major}-globin RNA (average, 43.5%).

Chromatin Structure. To determine whether hypersensitive sites were formed at their natural positions on the LAR derivatives after introduction into MEL cell lines, we analyzed the chromatin structure of the LAR/human β-globin gene cassettes in several of the MEL clones. Our results reveal that all four 5′ ε hypersensitive sites are formed in the stably integrated mLAR (Fig. 3*A*) and μLAR (data not shown) β-globin gene cassettes. In addition, these sites are formed both prior to and after induction of the MEL cells. Analysis of the chromatin structure of the 6,11 and 15,18 LAR reductions (Fig. 3 *B* and *C*) revealed that the appropriate hypersensitive sites are formed in uninduced MEL cells. These results reinforce the correlation between activity of the LAR elements and the formation of these alterations in chromatin structure. We have also observed additional subbands corresponding to hypersensitive sites in flanking mouse DNA in several MEL clones containing the m6,11 and μ6,11 β-globin cassettes (Fig. 3*B* and data not shown). At this time, we do not know the significance of these hypersensitive sites.

Tissue Specificity of the LAR Effect. The tissue specificity of the LAR effect was studied by introducing the human β-globin gene described above, with and without the mLAR, into NIH 3T3 cells by electroporation. In the absence of the LAR, the β-globin gene is transcribed, but the levels are variable and do not correlate with the number of β-globin gene copies (Fig. 4*A*). This result is consistent with previous observations regarding the activity of β-globin genes introduced into nonerythroid cells by transfection (21). When the mLAR is linked to the human β-globin gene in either the genomic (LAR; Fig. 4*A*) or reverse genomic orientation (data not shown) and introduced into 3T3 cells, no effect on the steady-state levels of human β-globin RNA is observed. Analysis of the chromatin structure of the LAR sequences in mLAR/β-globin cassette containing 3T3 clones reveals that a hypersensitive site is formed at the position of the previously described erythroid specific hypersensitive site located −10.9 kb to the ε-globin gene (Fig. 4*B*). This hypersensitive site was formed equally well in the mLAR in both orientations (data not shown).

DISCUSSION

The results presented above show that the sequences retained after reduction of the 5′ ε region from 20 to 2.5 kb (μLAR) confer orientation-independent high-level expression of a linked β-globin gene in stably transformed MEL cells. We have also demonstrated that as little as 1500 base pairs of the μLAR (μ6,11) and a cassette containing the −15 and −18 hypersensitive site regions significantly increased β-globin gene expression in MEL cells. However, the two "half" LARs are not as active as the complete mLAR or

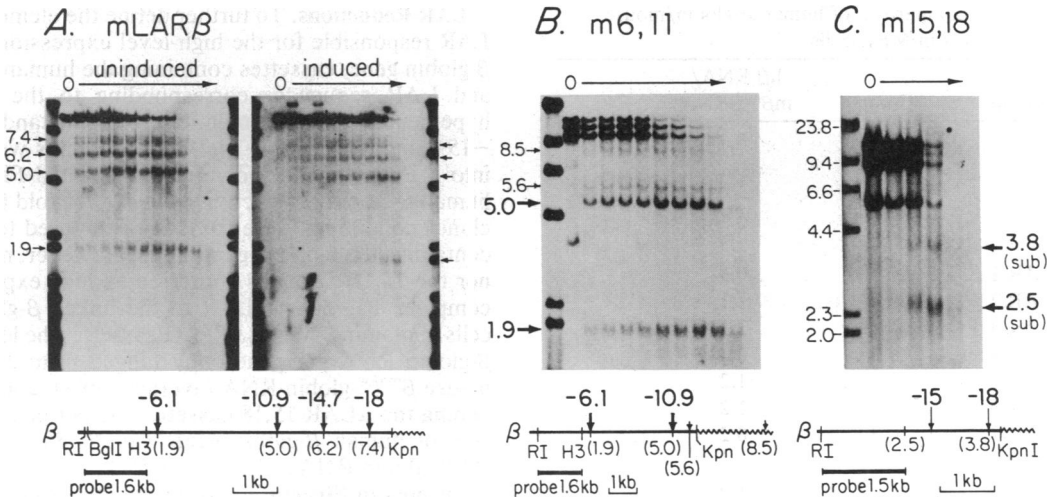

FIG. 3. Chromatin structure of mini-LAR (*A*), mini6,11 (*B*), and mini15,18 (*C*) in MEL cells. Analysis was performed on uninduced and induced cells containing a single mLAR insert (*A*), uninduced cells containing three m6,11 inserts (*B*), and uninduced cells containing four m15,18 inserts (*C*). DNA from DNase I-treated nuclei was digested with *Eco*RI, electrophoresed, blotted, and hybridized with the 1.6-kb *Eco*RI/*Hin*dIII probe. Positions of DNase I hypersensitive sites within the LAR derivatives are shown in kb from the *Eco*RI site in parentheses beneath the original genomic location of these hypersensitive sites relative to the ε-globin gene. Wavy line indicates flanking cellular DNA. Size markers are λ/*Hin*dIII and φX/*Hae* III end-labeled restriction fragments. In *B*, prominent hypersensitive sites are shown with large arrows in left margin and correspond to the previously characterized −6.1 and −10.9 sites. Minor subbands are indicated with small arrows and are located near the junction of LAR sequences and mouse genomic DNA and within mouse DNA as shown on the map. Bands seen in the first, fifth, and sixth lanes of *B* are believed to be spurious and are not seen reproducibly.

μLAR. This result suggests that while the active elements within the LAR can function independently, these elements may work cooperatively or additively and may have separable functions in influencing the level of expression of the linked β-globin gene. The demonstration that small fragments containing LAR elements can effectively increase the level of a linked β-globin gene has led to the construction of retroviral vectors that are capable of significantly increased β-globin expression in MEL cells (U.N. and W.C.F., unpublished data).

The effect of the LAR sequences on β-globin gene expression is tissue specific: in 3T3 cells, LAR/β-globin gene cassettes are expressed at a low level, similar to the expression of the β-globin gene without LAR elements. However, in contrast to the absence of 5′ ε LAR hypersensitive sites in the chromatin of nonerythroid cells (7), the −11 hypersensitive site is formed on the LAR/β-globin gene cassette stably integrated in 3T3 cells. This unexpected result may be analogous to the finding that the transfected, but not the endogenous, β-globin gene is expressed in 3T3 and other nonerythroid cells and may reflect differences between the regulation of endogenous sequences and those that integrate into the genome after transfection and transient activity. The formation of the −11 hypersensitive site on the LAR in 3T3 cells suggests that at least a subset of the factors important in the generation of this hypersensitive site is present in these nonerythroid cells, but that this subset is not sufficient for high level β-globin gene expression.

Our chromatin analyses also revealed that all four DNase I hypersensitive sites are formed at the expected *in vivo* positions on all LAR derivatives before and after MEL cell induction and independent of the orientation of the LAR sequences with respect to the β-globin gene. The formation of these hypersensitive sites prior to induction of MEL cells is consistent with our previous findings that the human β-globin domain is DNase I sensitive and the LAR hypersensitive sites are present in K562 cells and in the human/MEL hybrids prior to induction and overt transcription of the globin genes (2, 3, 7). We have referred to this active state of chromatin present in cells prior to transcription as a "pre-

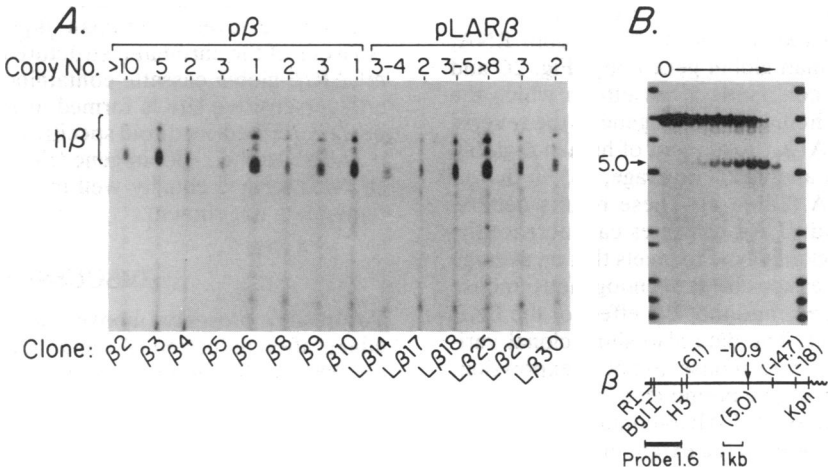

FIG. 4. Expression of the human β-globin gene plus/minus the LAR in 3T3 cells and the chromatin structure of the LAR in these cells. (*A*) Five micrograms of total RNA was assayed by RNase protection as described for Fig. 2. (*B*) Chromatin was analyzed as for Fig. 3.

activation" state (22–24) and have suggested that genes residing within a preactivation chromatin structure may have increased accessibility to gene-specific (i.e., promoter, enhancer, etc.) factors dependent on the induced state for their activity.

Sequence analysis of the μLAR constituent elements reveals that each of the four hypersensitive site regions contains enhancer core-like sequences and significant stretches of alternating purines and pyrimidines (4). In addition, the μ6 hypersensitive site region contains an oligo(T) stretch, and the μ15 element contains three consensus NFE-1 (ERYF-1) binding sites (25, 26). Thus, all hypersensitive site elements of the μLAR contain sequences that may be involved in the generation of altered DNA structures and/or transcriptional regulation. Recently, Driscoll *et al.* (27) have described a new deletional $(\gamma\delta\beta)^0$-thalassemia, in which sequences corresponding to the −6.1 hypersensitive site are retained 3′ to the breakpoint region, and the −11, −15, and −18 region are all deleted. This deletion suggests that sequences involved in the formation of the −6.1 hypersensitive site are not sufficient by themselves to influence the activation of the cis-linked β-globin locus. Consistent with this, we have found that the −6.1 hypersensitive site is not formed and the entire β-globin locus on the deleted chromosome is DNase I resistant in somatic cell hybrids resulting from the fusion of MEL cells and the lymphoid cells of this thalassemic patient (unpublished results). When considered along with this deletion, our results using the m6,11 cassette and those of Ryan *et al.* (28), who have recently reported that a cassette containing the −6 and −11 hypersensitive sites confers high level expression to a linked α-globin gene in transgenic mice, suggest either that the −11 and −6 elements must interact or that the −11 region alone is sufficient to confer a functional LAR effect. In addition, the high-level expression conferred to the α-globin gene by the β-globin LAR implies that the α-globin locus may be regulated by a similar LAR mechanism.

Grosveld *et al.* reported originally that cassettes containing the LAR sequences and the 3′ β developmentally stable hypersensitive site region conferred copy-number-dependent, position-independent expression of a linked β-globin gene in transgenic mice (9). However, our results in clonal MEL lines and those of Ryan *et al.* (29) in transgenic mice have revealed significant variation in the per copy expression of LAR-linked β-globin genes. Although we did not include the 3′ β developmentally stable hypersensitive site in our cassettes, others have observed significant variability using constructs that included this region (29, 30).These studies were performed in MEL cells (30) as well as in transgenic mice (29, 30), eliminating the possibility that variability is a cell line artifact. The basis for these discrepancies may reside within the methods of analysis in the different studies. For example, we and van Assendelft (30) analyzed clonal lines of transfected MEL cells and observed substantial variability in LAR-associated β-globin gene expression among different clones. However, analyses of pooled populations of transfected MEL cells have not demonstrated the same variability in LAR-associated β-globin gene expression (30, 31). The differences observed in the various transgenic experiments are more difficult to explain (9, 29) and raise the question of whether the LAR elements confer absolute position-independent expression to linked genes.

It has also been reported recently that the −10.9-kb component of the LAR can function as a classic enhancer in transient assays (32). However, it remains to be determined whether any LAR sequences function as enhancers when located in their natural chromosomal position >50 kb from the β-globin gene. One model that could explain the concentration of NFE-1 (ERYF-1) binding sites, core enhancer, and other unusual DNA sequences within the LAR is that such sequences may serve as sites for the displacement of nucleosomes associated with the binding of other factors, thereby resulting in stable alterations in the topology of the β-globin locus in erythroid cells.

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