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High-level β-globin expression after retroviral transfer of locus activation region-containing human β-globin gene derivatives into murine erythroleukemia cells

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ABSTRACT **The locus activation region (LAR) of the human β-globin-like gene cluster is characterized by a group of four DNase I hypersensitive sites, which arise specifically in erythroid tissues and are required for a normal pattern of β-globin-like gene expression. The hypersensitive sites are found at positions 6.1, 10.9, 14.7, and 18 kilobase pairs (kbp) 5′ of the ε-globin gene. Recently functional assays of the LAR that tested determinants for all four hypersensitive sites showed that expression of the human β-globin gene was increased to normal or near-normal levels in both transgenic mice and erythroid cells. We constructed retroviral vectors with a human β-globin gene and the determinant for a single hypersensitive site and measured β-globin gene expression after retroviral infection of murine erythroleukemia cells. Fragments for the hypersensitive sites at −18 or −10.9 kbp increased human β-globin RNA levels respectively to 35% or 132% of the endogenous mouse β^{maj}-globin RNA level. In addition, greater expression was also observed for the neomycin phosphotransferase RNA, which was transcribed from the retroviral LTR, showing that the LAR fragments activated expression from a heterologous promoter. In the context of gene-transfer experiments ultimately aimed at gene therapy, our results show that LAR determinants lead to an increased level of human β-globin RNA expression after retroviral transfer into erythroid cells. But inclusion of LAR determinants in retroviral vectors also entails the potential risk of activating the expression of non-globin genes in erythroid cells.**

To determine if gene-addition therapy for β-thalassemia and sickle cell anemia is feasible, several criteria must be met regarding the efficiency of gene transfer into bone marrow stem cells and the stringency of β-globin gene regulation. Replication-deficient retroviral vectors represent a promising option for transfer of a β-globin gene into bone marrow stem cells (1). Human and mouse bone marrow cells have been infected successfully with retroviruses (2–6), and retroviral vectors have been used (7, 8) to transfer the human β-globin gene into mouse bone marrow cells. Mice reconstituted with the infected bone marrow showed tissue-specific human β-globin protein and RNA expression, and the infected marrow resulted in long-term β-globin expression after transfer to additional mice in serial transplantation experiments. This indicated that pluripotent hematopoietic stem cells rather than committed progenitor cells were infected (7, 8). But in both of these virus-mediated β-globin gene-transfer experiments, the transduced human β-globin gene was expressed at a low level (1–2%) compared with the endogenous mouse β^{maj}-globin gene (7, 8). Our work is focused on achieving a normal level of expression for the virally transferred human β-globin gene.

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Studies of a "mini β-globin gene locus" in transgenic mice revealed the importance of the 20-kilobase-pair (kbp) region of the human β-globin-like gene cluster that lies 5′ to the ε-globin gene for high-level β-globin gene expression (9). This region is characterized by four developmentally stable DNase I hypersensitive sites, which map 6.1, 10.9, 14.7, and 18.0 kbp 5′ of the ε-globin gene in human erythroid tissues or cell lines in which any of the β-globin-like genes are expressed (9–12). These hypersensitive sites form before overt expression of the β-globin genes, as shown by studies of somatic hybrids between human lymphocytes and murine erythroleukemia (MEL) cells (12), which suggests that events that occur initially in the hypersensitive site region may be necessary for the subsequent expression of the β-globin genes. In addition, since naturally occurring deletions that remove these hypersensitive sites leave the adjacent β-globin locus transcriptionally inactive (13, 14), this region has been called the "locus activation region" (LAR) (12). It is now clear that the LAR can be reduced in size from 18 kbp to 2.5 kbp (15) or 6.5 kbp (16) while retaining the ability to direct high-level expression of a β-globin reporter gene after transfer to MEL cells. Here we report that the individual hypersensitive sites differ in the ability to increase the level of steady-state β-globin RNA. Whereas the determinant for the hypersensitive site at −6.1 kbp had no effect on β-globin RNA expression, determinants for the other three hypersensitive sites led to increased human β-globin expression. The implications of the data for the use of LAR-containing β-globin gene retroviral vectors in gene therapy protocols are discussed.

MATERIALS AND METHODS

Cell Culture. We have described (6, 17) sources and culture conditions for ψ-2 and PA317 retrovirus packaging cell lines, the adenine phosphoribosyltransferase-negative mutant of MEL cells, and the thymidine kinase-negative mutant of NIH 3T3 cells. Cells expressing neomycin phosphotransferase activity were selected by addition of 1–1.5 mg of G418 per ml of which about 50% was active. Clones of G418-resistant MEL cells were obtained by plating in medium containing 2.5 mg of Bacto agar and 1–1.5 mg of G418 per ml. Colonies were removed after 12–20 days and expanded in liquid cultures.

Generation of Retroviruses and Infection of MEL Cells. All retrovirus vectors in this report were based on the vector LNβ*SA (17), which contains a neomycin phosphotransferase gene, *neo*, under the control of the promoter and enhancer sequences of the long terminal repeat (LTR) of the Moloney murine leukemia virus (Mo-MLV) and a human

Abbreviations: LAR, locus activation region; MEL, murine erythroleukemia; LTR, long terminal repeat; Mo-MLV, Moloney murine leukemia virus.

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β-globin gene. The human β-globin gene contains sequences from base pair (bp) −615 (*Sph* I site) through +2482 (*Avr* II site) and is marked by a 6-bp insertion in the 5′ untranslated region (6). The marked β-globin gene is inserted between *neo* and the 3′ LTR and is transcribed in the opposite direction relative to transcription from the 5′ LTR (Fig. 1). Cleavage sites for *Hin*dIII and *Hpa* I, which were used for the construction of the LAR derivatives, are located 5′ of the human β-globin gene but before the 3′ LTR. Fragment 18s (s = small), which contained the hypersensitive site at −18 kbp, was a 487-bp *Hae* III fragment located between 1195 and 1682 on the published DNA sequence of the human β-globin-like gene cluster (GenBank HUMHBB, accession no. J00179). Fragment 15s contained the hypersensitive site at −14.7 kbp and was cloned into the vector as a 569-bp *Hin*dIII/*Bal* I fragment (positions 4610–5172). The site at −10.9 kbp was cloned as a 2-kbp *Hin*dIII fragment (8,486–10,411; fragment 11) or as a 1267-bp *Hin*dIII/*Pvu* II fragment (fragment 11s). The hypersensitive site at −6.1 kbp was cloned as a 1.4-bp *Hin*dIII fragment (12,398–13,769; fragment 6) or as a 761-bp *Rsa* I fragment (12,932–13,693; fragment 6s). The *Hin*dIII sites of the fragments were cloned into the *Hin*dIII site of the vector; the *Bal* I, *Pvu* II, or *Rsa* I sites were cloned into the *Hpa* I site of the vector. A construct that contained the hypersensitive sites at −14.7 kbp and −18.0 kbp as a 1.0-kb *Hin*dIII fragment (designated μ15,18) was derived from plasmids described elsewhere (15).

Retroviruses were generated as described (17). Briefly, supercoiled plasmids were transfected into the ecotropic packaging cell line ψ-2. Transiently expressed virus was harvested after 2 days and used to infect the amphotropic retrovirus packaging cell line PA317, which was plated in G418-containing medium. The structure of the integrated provirus in clonal retrovirus-producing cell lines was analyzed by digestion of producer line DNA with *Kpn* I, which cuts the provirus in each LTR, and Southern blot analysis with a *neo* probe. MEL cells were infected with supernatant medium from amphotropic retrovirus-producing cell lines (6).

RNase Protection Analysis of Globin and neo RNA. RNA was prepared by lysis of the cells with guanidine isothiocyanate and subsequent selective LiCl precipitation (18). Steady-state levels of human β-globin or mouse β^{maj}- and β^{min}-globin RNA as well as neo mRNA were determined by RNase-protection assays utilizing probes that detected 5′-terminal fragments of human or mouse β-globin RNA as described (6). Neo RNA was measured with an RNA probe that protected an internal fragment of the neo transcript from the *Nru* I site to the *Pst* I site, 119 nucleotides long. The relative amounts of human and mouse β-globin were quantitated by assaying radioactivity in the gel slices containing the protected RNA fragments after autoradiography of the gel and correcting for background and the number of uridine residues in the respective mouse and human probes.

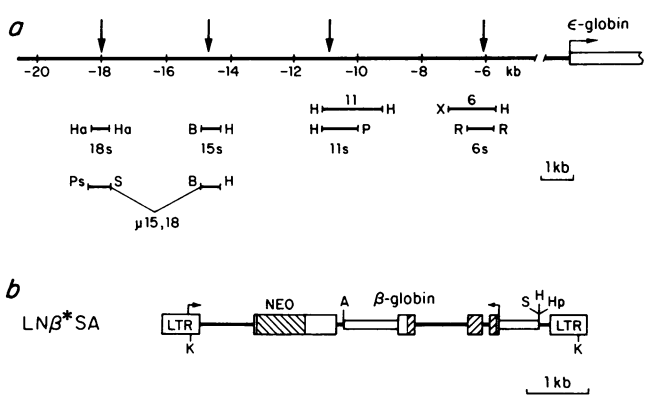

FIG. 1. (*a*) Map of the LAR sequences used for retrovirus constructions. Locations of the DNase I hypersensitive sites upstream of the ε-globin gene are indicated by arrows. Restriction fragments inserted in the viral vectors are shown. B, *Bal* I; H, *Hin*dIII; Ha, *Hae* III; P, *Pvu* II; Ps, *Pst* I; R, *Rsa* I; S, *Sph* I; X, *Xba* I. (*b*) Map of the retrovirus LNβ*SA. The position of *neo*, its transcriptional start site in the LTR, and the reverse-orientation human β-globin gene within the virus are shown. Hatched boxes are coding sequences, open boxes are noncoding sequences, and solid bars show locations of the introns in the globin gene. Cloning sites used for the insertion of LAR fragments (S = *Sph* I, H = *Hin*dIII, and Hp = *Hpa* I) and the *Kpn* I sites (K) in the LTRs are shown.

Detection of DNase I Hypersensitive Sites in Isolated Nuclei. Methods for isolation of nuclei from MEL cells (12) and for DNase I digestions (19) have been described. DNA was isolated from DNase I-treated nuclei, digested with *Eco*RI, subjected to electrophoresis in 0.7% agarose gels, blotted, and hybridized with a *Bam*HI–*Eco*RI fragment that contained the large intervening sequence of the human β-globin gene.

RESULTS

Production of β-Globin Gene-Transducing Viruses with LAR Fragments. To test if LAR fragments for single hypersensitive sites would be compatible with virus replication and would lead to increased β-globin RNA expression, we first ligated 1.3- or 2.0-kbp fragments specific for the hypersensitive sites located at 6.1 kbp (designated "6" in vector names) or 10.9 kbp (designated "11" in vector names) upstream of the ε-globin gene into the β-globin gene-transducing vector LNβ*SA as shown in Fig. 1. Unfortunately, cell lines made with the LNβ*SA-6 and LNβ*SA-11 constructs showed large internal deletions encompassing not only the inserted fragment but most, if not all, of the β-globin gene as well (data not shown). Subsequent derivatives of LNβ*SA contained smaller fragments, which are denoted with the suffix "s." Fragments for the hypersensitive sites that map 14.7 or 18 kbp upstream of the ε-globin gene were designated "15" or "18" in vector names. The LNβ*SA-6s, LNβ*SA-11s, LNβ*SA-15s, LNβ*SA-18s viruses contained the indicated small LAR fragment in the opposite genomic orientation relative to the β-globin gene (Fig. 1). The LNβ*SA-μ15,18 virus carried a 1-kbp fragment with determinants for both hypersensitive sites at 15 and 18 kbp in the forward orientation relative to the β-globin gene. Stable packaging cell lines were obtained with unrearranged proviruses for each of the small LAR fragments, although these were in the minority. The titers exhibited by most of these cell lines ranged between 2×10^4 and 10^5 colony-forming units/ml, which were adequate for infection of MEL cells and measurement of β-globin expression. More details on these cell lines will be published elsewhere.

Packaging cell lines for the 6s, 15s, and 18s derivatives of LNβ*SA produced virus that faithfully transmitted unrearranged copies to MEL cells based on Southern blot analysis (data not shown). But packaging cell lines for the 11s or the μ15,18 derivatives of LNβ*SA, despite initially harboring unrearranged proviruses, produced only 43% or 68% correct progeny viruses, respectively. Thus, the tendency of some of these lines to produce rearranged viruses necessitated confirmation of the structure of the integrated provirus in clonally infected MEL lines in subsequent experiments.

Expression of Human β-Globin RNA in MEL Cells. Expression of the LAR-containing β-globin gene derivatives was studied by infection of MEL cells. MEL cells are arrested at a late stage in erythroid development, but gene expression characteristic of terminal erythroid differentiation can be induced in culture (20). Individual clones were isolated after culturing the cells in G418-containing semisolid medium. Both the structure of the integrated proviral DNA sequences and the pattern of proviral integration were determined by

Southern blotting for all clonal cell lines (data not shown). Only those G418-resistant MEL cell lines that carried single copies of the unrearranged provirus and that showed a unique site of integration in the MEL cell DNA were included in the analysis of β-globin expression.

Steady-state levels of human β-globin and mouse β^{maj}-globin RNA were measured by RNase protection assays in RNA samples from MEL cells that were infected with the control LNβ*SA virus or its 6s, 11s, 15s, 18s, or μ15,18 derivatives. Fig. 2 shows the pattern of correctly initiated human and mouse β^{maj}-globin RNA from some representative clonal MEL cell lines. More data will be published elsewhere. After 6 days of treatment with 2% dimethyl sulfoxide, induction of mouse β^{maj}-globin RNA was consistently high. The amount of human β-globin RNA after induction, expressed as a percentage of the mouse β^{maj}-globin RNA in the same sample, is given in Table 1 for the cell lines shown in Fig. 2 along with data from some additional cell lines. The amounts of human or mouse β-globin RNA after induction divided by the uninduced RNA level, which is a measure of the extent of induction, are also presented in Table 1. Human β-globin RNA induction was not affected in most cases by the addition of fragments from the LAR to the LNβ*SA vector and ranged from 2- to 35-fold. For the LNβ*SA-11s provirus, low levels of human β-globin RNA before induction taken with the high RNA levels attained after induction resulted in a 38- to 643-fold induction range. Induction of mouse β^{maj}-globin RNA ranged from 13- to 370-fold.

The parental LNβ*SA virus directed expression of the human β-globin RNA in induced MEL cells at levels between 1% and 11% (averaging 5.7%) of the endogenous mouse β^{maj}-globin RNA in eight cell lines (Fig. 2*a* and Table 1). Similar levels of 2% to 24% (averaging 7.5%) were observed in MEL clones infected with viruses that contained the 6s fragment (Table 1). In contrast, the LNβ*SA-11s provirus expressed human β-globin RNA at 10% to 310% (averaging 132%) of the endogenous level (Table 1). Proviruses that contained individual 15s or 18s fragments expressed human β-globin RNA at steady-state levels between 5.8% and 55% (averaging 24%) or between 10% and 75% (averaging 35%), respectively, of the endogenous levels (Fig. 2 *b* and *c*). Surprisingly, the LNβ*SA-μ15,18 virus, which carried determinants for both of these hypersensitive sites, directed human β-globin RNA expression that ranged from 0.8% to 79% (averaging 20%) of the endogenous level (Table 1). The ratios of human to mouse β-globin RNA summarized in Table 1 represent conservative estimates of expression from the virally transferred globin genes, since no correction was made for the mouse β-globin gene copy number, which may have been higher than one per cell.

Alterations in Chromatin Structure in Virus-Infected Clonal MEL Cells. We investigated whether DNase I hypersensitive sites would be formed in the chromatin of MEL clonal lines that were infected with LNβ*SA-6s, -15s, -18s, and -μ15,18 viruses. Nuclei were purified from uninduced MEL cells and subjected to digestion with DNase I at a range of concentrations. DNase I hypersensitive sites formed on the 15s and 18s fragments present in their respective integrated proviruses (Fig. 3). In the LNβ*SA-18s virus, DNase I cleavage at the closely spaced hypersensitive sites located in the LAR fragment and the strong transcriptional enhancer in the 3′ LTR of the virus resulted in subbands that were incompletely resolved in this experiment. The 6s fragment also directed the formation of a hypersensitive site (data not shown), although there was little if any effect on β-globin expression. MEL cells infected with the LNβ*SA-μ15,18 provirus showed only one hypersensitive site which mapped to the 18s portion of the μ15,18 fragment. Hypersensitive sites that mapped to the 3′ LTR of the integrated provirus and the promoter of the human β-globin gene were also present.

LAR Effects on Nonglobin Gene Expression. Since the retroviral vectors used in this study transduced the *neo* gene as well as the human β-globin gene, neo RNA levels were measured to determine if the various fragments derived from the LAR would influence an RNA transcribed from the nonerythroid Mo-MLV promoter. A low level of neo RNA, which showed a slight response to induction in some MEL clones, was observed for several LNβ*SA-infected MEL clones (Fig. 4). In contrast, the level of neo RNA increased dramatically in response to induction in the MEL clonal lines that had been infected with the LNβ*SA-6s, -15s, and -18s derivatives. The steady-state neo RNA level increased 5- to 50-fold in the LAR derivatives of the LNβ*SA virus (data

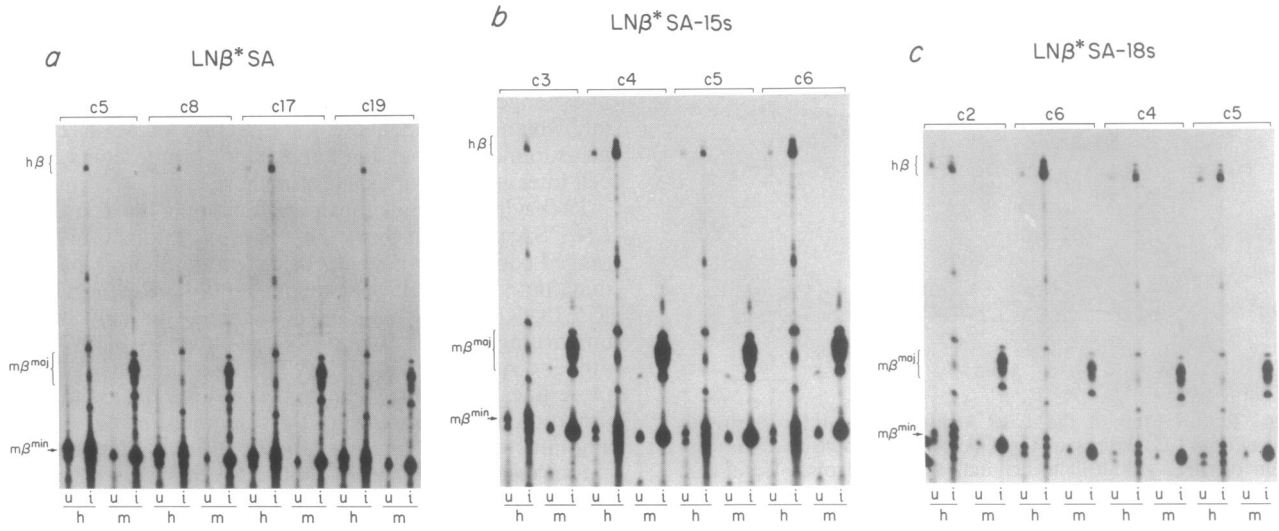

FIG. 2. Expression of human β-globin in infected MEL cells. Levels of human and mouse globin RNA were determined by an RNase protection assay. RNA (3 μg) from uninduced (lanes u) or induced (lanes i) clonal lines of MEL cells infected with LNβ*SA (*a*), LNβ*SA-15s (*b*), or LNβ*SA-18s (*c*) viruses were hybridized with uniformly labeled RNA probes spanning the human (lanes h) or mouse (lanes m) β-globin RNA cap sites. The predominant fragment protected by the human β-globin probe was 138 nucleotides (nt), corresponding to correctly initiated transcripts (hβ). The mouse β^{maj}-globin probe protected fragments of about 60 nt derived from mouse β^{maj}-globin RNA (β^{maj}) and 43 nt derived from mouse β^{min}-globin RNA (β^{min}).

Table 1. Globin mRNA induction and relative expression levels in infected MEL cell clones

Provirus; MEL clone number	β-Globin RNA induction		Human/mouse ratio × 100,‡ %
	Mouse β^{maj}*	Human†	
LNβ*SA;c1	54	2.6	11.0
LNβ*SA;c3	370	5.6	1.2
LNβ*SA;c4	68	11.0	7.7
LNβ*SA;c5	67	7.8	4.2
LNβ*SA;c8	50	2.8	2.6
LNβ*SA;c11	21	1.7	1.4
LNβ*SA;c17	79	5.1	8.1
LNβ*SA;c19	23	14.0	9.4
LNβ*SA-6s;c1	150	2.8	2.0
LNβ*SA-6s;c2	100	13.0	4.1
LNβ*SA-6s;c3	240	6.3	7.0
LNβ*SA-6s;c4	230	2.5	2.3
LNβ*SA-6s;c5	91	2.0	3.9
LNβ*SA-6s;c7	120	3.7	9.5
LNβ*SA-6s;c8	130	12.0	24.0
LNβ*SA-11s;c102	13	190.0	78.0
LNβ*SA-11s;c103	63	38.0	10.0
LNβ*SA-11s;c105	31	643.0	310.0
LNβ*SA-15s;c3	94	7.1	5.8
LNβ*SA-15s;c4	79	25.0	27.0
LNβ*SA-15s;c5	170	30.0	13.0
LNβ*SA-15s;c6	79	4.7	22.0
LNβ*SA-15s;c7	13	3.6	55.0
LNβ*SA-15s;c8	110	10.0	51.0
LNβ*SA-15s;c9	31	5.6	21.0
LNβ*SA-18s;c2	90	2.1	12.0
LNβ*SA-18s;c4	46	6.2	25.0
LNβ*SA-18s;c5	56	6.0	40.0
LNβ*SA-18s;c6	13	3.3	75.0
LNβ*SA-18s;c7	35	10.0	18.0
LNβ*SA-18s;c8	41	5.7	66.0
LNβ*SA-18s;c9	100	8.6	10.0
LNβ*SA-μ15,18;c14	21	2.90	79.00
LNβ*SA-μ15,18;c17	190	2.30	0.76
LNβ*SA-μ15,18;c29	180	4.20	3.60
LNβ*SA-μ15,18;c30	54	10.40	38.00
LNβ*SA-μ15,18;c32	51	26.30	21.00
LNβ*SA-μ15,18;c33	54	3.93	2.40
LNβ*SA-μ15,18;c34	51	5.87	3.80
LNβ*SA-μ15,18;c35	120	13.00	13.00

*Ratio of mouse β^{maj}-globin RNA cpm in induced cells to mouse β^{maj}-globin RNA cpm in uninduced cells.
†Ratio of human β-globin RNA cpm in induced cells to human β-globin RNA cpm in uninduced cells.
‡Ratio of human β-globin RNA cpm to mouse β^{maj}-globin RNA cpm (corrected for the probe compositions), expressed as a percentage.

not shown). The degree of induction was similar regardless of which LAR fragment was present in the provirus. Quantitative results will be presented elsewhere. The uniformity of action of the 6s, 15s, and 18s fragments in leading to more stringent erythroid-like regulation of neo RNA expression is interesting in view of the inability of the 6s fragment to influence human β-globin expression in the same construct.

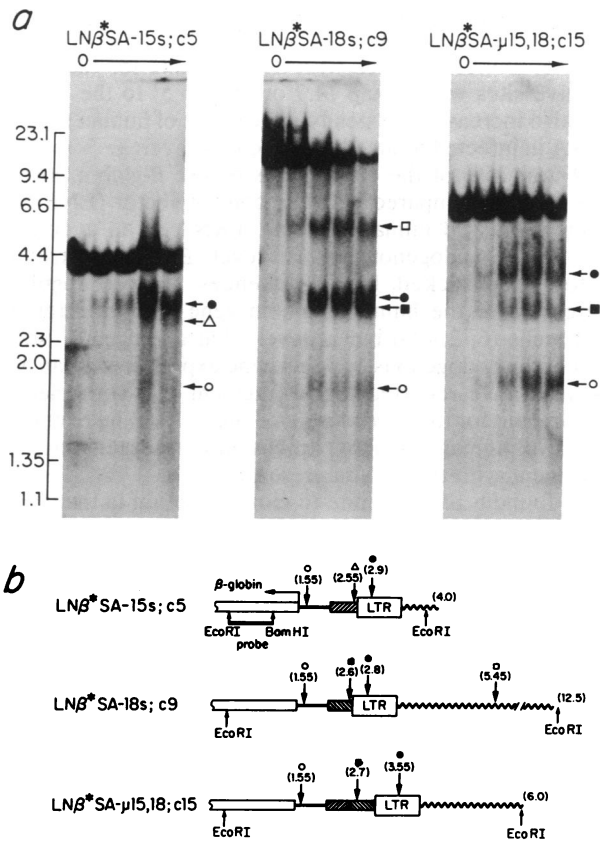

FIG. 3. (*a*) Chromatin analysis of infected MEL cells. Aliquots of nuclei of three clones, LNβ*SA-15s;c5, LNβ*SA-18s;c9, and LNβ*SA-μ15,18;c15, were treated with increasing amounts of DNase I as shown above each panel. ○, Position of the hypersensitive sites 5′ of the β-globin gene; △, position of the hypersensitive site at −15 kbp; ■, position of the hypersensitive site at −18 kbp; ●, position of the hypersensitive site in the LTR; and □, position of an additional hypersensitive site in cell line LNβ*SA-18s;c9. Size markers shown at the left are phage λ *Hin*dIII fragments and $\phi\chi174$ *Hae* III end-labeled restriction fragments. (*b*) Distance of the hypersensitive site in the three clones from the *Eco*RI site in the human β-globin gene is shown in parentheses. Hatched sections indicate the LAR sequences contained in the integrated viruses. Wavy lines represent flanking genomic DNA.

DISCUSSION

We prepared retrovirus vectors with the human β-globin gene along with the determinants for individual hypersensitive sites from the LAR of the human β-globin-like gene cluster in an attempt to increase globin gene expression from single integrated provirus copies. β-Globin gene expression was studied in clonal erythroid cell lines after confirming the presence of single proviral insertions at unique positions, such as might be expected after virus-mediated gene transfer to hematopoietic stem cells. One vector (LNβ*SA-11s) di-

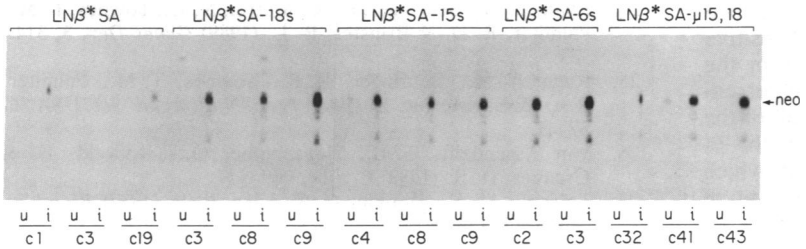

FIG. 4. Effect of LAR sequences on *neo* expression. Levels of neo RNA in several uninduced (lanes u) and induced (lanes i) MEL cell clones infected with LNβ*SA, LNβ*SA-18s, LNβ*SA-15s, LNβ*SA-6s, and LNβ*SA-μ15,18 were assayed by RNase protection. RNA (3 μg) was analyzed after hybridization to a uniformly labeled RNA fragment containing sequences of the 3′ untranslated region of *neo*.

rected human β-globin RNA expression at a level comparable to the endogenous mouse β^{maj}-globin RNA level in a small group of clones. Viruses with determinants for the hypersensitive sites which map 14.7 or 18 kbp 5′ to the ε-globin gene also increased the steady-state levels of human β-globin mRNA in infected induced MEL cells on average 5- to 7-fold to 24% or 35% of the endogenous mouse β-globin mRNA, respectively, compared with the control vector (LNβ*SA), which expressed human β-globin mRNA at an average of 5.7% of the endogenous mouse level. β-Globin retrovirus vectors that lacked LAR sequences showed regulated expression of the human β-globin gene after transfer into MEL cells (6, 21–23) but at levels that averaged 5–10% at most of the endogenous β-globin gene expression. Ryan *et al.* (24) also have reported that a fragment that contained the determinant for the hypersensitive site at 10.9 kbp resulted in high-level human β-globin expression in transgenic mice, in agreement with the results presented here.

We found a 10-fold clone-to-clone variation in the level of human β-globin to mouse β^{maj}-globin RNA for the control LNβ*SA virus and from 7.5-fold to >30-fold for its LAR derivatives. Although variations in the extent of erythroid induction may have contributed to these differences, most of the variation probably reflects effects of the neighboring DNA sequences on the transcription of the different proviral insertions or position effects. Thus, the attribute of position-independent expression, which was described for the original minilocus in transgenic mice (9) and more recently for a 6.5-kbp derivative as tested in transgenic mice or in large polyclonal populations of MEL cells (16), may only be a property of all four hypersensitive sites when present in tandem arrays or of LAR sequences not present in the fragments we tested. Strict position-independent expression has not been observed in studies of the LAR from other laboratories. Ryan *et al.* (24, 25) studied constructs similar to those originally reported by Grosveld *et al.* (9) in transgenic mice and observed increased expression of human α- or β-globin genes, but the pattern of expression was neither strictly copy number dependent nor position independent. In other studies of stably transformed MEL cells, the ratio of human β-globin to mouse β-globin RNA expression varied 5- to 6-fold with 8-kbp or 2.5-kbp forms of the LAR, which included all four DNase I hypersensitive sites (15), or >16-fold with the original minilocus construct (26).

Interestingly, viral transfer of a 1-kbp fragment with determinants for hypersensitive sites at both −18 and −14.7 kbp had less influence on β-globin RNA expression (20% of the mouse β^{maj}-globin level) than when either of these sites was tested individually. In contrast, the same hypersensitive site determinants present on a larger 2.3-kbp fragment cause a linked β-globin gene to be expressed at >40% of the endogenous mouse β^{maj}-globin level after transfer to MEL cells by electroporation (15). Conceivably, the fragments chosen for the smaller construct interfered with LAR function, or perhaps the hypersensitive sites at −14.7 and −18.0 kbp may show negative cooperativity if placed too close together.

These differences in biological activity, revealed for the individual fragments that determine the hypersensitive sites at −18, −14.7, −10.9, and −6.1 kbp, are consistent with the phenotype of a naturally occurring deletion that eliminates most of the LAR. In a form of $(\gamma\delta\beta)^0$-thalassemia described by Driscoll *et al.* (27), DNA that includes the hypersensitive sites at −18, −14.7, and −10.9 kbp is deleted from the β-globin-like gene cluster, but sequences for the hypersensitive site at −6.1 kbp and 3′ sequences including all of the β-globin-like genes are intact. No expression from the β-globin-like genes *cis* to this deletion can be detected, which suggests that the primary determinants for activation of β-globin gene expression lie upstream of the site at −6.1 kbp.

In vivo tests of the LNβ*SA-LAR vectors will be required to determine if a therapeutically useful level of RNA expression is achieved, since expression in MEL cells does not necessarily predict expression *in vivo*. The action of the LAR-derived fragments in increasing Mo-MLV *neo* expression after induction is a reminder of the potential for insertional activation of nonglobin genes, with potentially deleterious consequences from retroviral vectors of this type in gene addition protocols aimed at gene therapy.

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