DNaseI hypersensitive sites 1, 2 and 3 of the human β-globin dominant control region direct position-independent expression

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ABSTRACT

The human β-globin dominant control region (DCR) which flanks the multigene β-globin locus directs high level, site of integration independent, copy number dependent expression on a linked human β-globin gene in transgenic mice and stably transfected mouse erythroleukemia (MEL) cells. We have assayed each of the individual DNaseI hypersensitve regions present in the full 15kb DCR for position independence and copy number dependence of a linked β-globin gene in transgenic mice. The results show that at least three of the individual DNaseI hypersensitive site regions (sites 1, 2 and 3), though expressing at lower levels than the full DCR, are capable of position independent, copy number dependent expression. Site 2 alone directs the highest level of expression of the single site constructs, producing nearly 70% of the level of the full DCR. Sites 1 and 3 each provide 30% of the full activity. Deletion of either site 2 or 3 from the complete set significantly reduces the level of expression, but does not effect position independence or copy number dependence. This demonstrates that sites 2 and 3 are required for full expression and suggests that all the sites are required for the full expression of even a single gene from this multigene locus.

INTRODUCTION

The human β-like globin genes are a family of five, closely linked, active genes arranged in the order of their developmental expression on the short arm of chromosome 11 (Fig. 1). Progress in delineating and defining the function of cis-elements involved in transcriptional regulation of the human β-globin gene has been achieved through the use of transgenic mice and mouse erythroleukemia (MEL) cell model systems. Three tissue-specific regulatory regions, found within and flanking the gene, contain sufficient information for differentiation- and developmental-stage specific expression (1,2,3,4). These regions encompass the promoter and two downstream enhancer elements, located within the gene and in the 3′ flanking region. A number of cis-acting elements within these regions have been identified which bind ubiquitous and/or erythroid-specific proteins (5,6,7), the most notable being the erythroid factor NF-E1 (GF-1, Eryf1) which has been cloned from human (8), mouse (9) and chicken (10).

Human β-globin gene constructs which contain all these regions have been shown to result in tissue- and stage-specific expression. However, the level of expression of the exogenous gene is highly variable, not dependent on copy number, and occurs at a fraction of the level of its endogenous counterpart (1,11,12,13). These phenomena have been attributed to effects of the integration locus and suggested that insufficient sequence information was present for fully regulated, position independent expression. Our laboratory has shown that inclusion of the dominant control region (DCR), confers position independent, high level expression on a linked β-globin gene in MEL cells (14) and transgenic mice (15). The DCR which is contained in 20kb upstream of the β-globin cluster (Fig. 1a,b) consists of a series of four erythroid specific DNaseI hypersensitive regions designated 5′ to 3′, sites 1,2,3,4 (15) or IV, III, II, I (16), or −18, −14.7, −10.9 and −6.1 (17). Linkage of the four DNA fragments which span the individual hypersensitive sites into a micro DCR (Fig. 1c) retains full functional capability, regardless of its orientation relative to the linked β-globin gene (18). The DCR is also capable of inducing high level expression of α-globin (19,20), γ-globin (21,22) and heterologous genes (15,17).

It is important to point out that the DCR has been assayed in a number of expression systems with varying results, suggesting that the individual sites have different functional capabilities. Transient assays in K562 cells (23) and MEL cells (C-H. Chang and P. Dierks pers. comm.) have shown enhancer-like activity in site 3, but not in sites 1,2 or 4. Expression analysis in stably transformed MEL cell populations has revealed that both sites 2 and 3 are capable of directing high levels of inducible β-globin expression, while sites 1 and 4 give lower but significant levels of activity (24). These results suggest that sites 1, 2 and 4 require integration into chromatin for their activity to be detected. Results obtained with a DCR deletion construct in transgenic mice suggested that at least site 3 can activate the β-globin gene, although it was not clear to what level and whether this provided position independence (20,25).

In this paper we assess the activity of each of the individual DNaseI hypersensitive sites (sites 1−4) on a linked β-globin gene in transgenic mice. In addition we analyze the expression of DCR constructs in which sites 2 or 3 have been deleted from the full microlocus construct. The results show that the individual sites

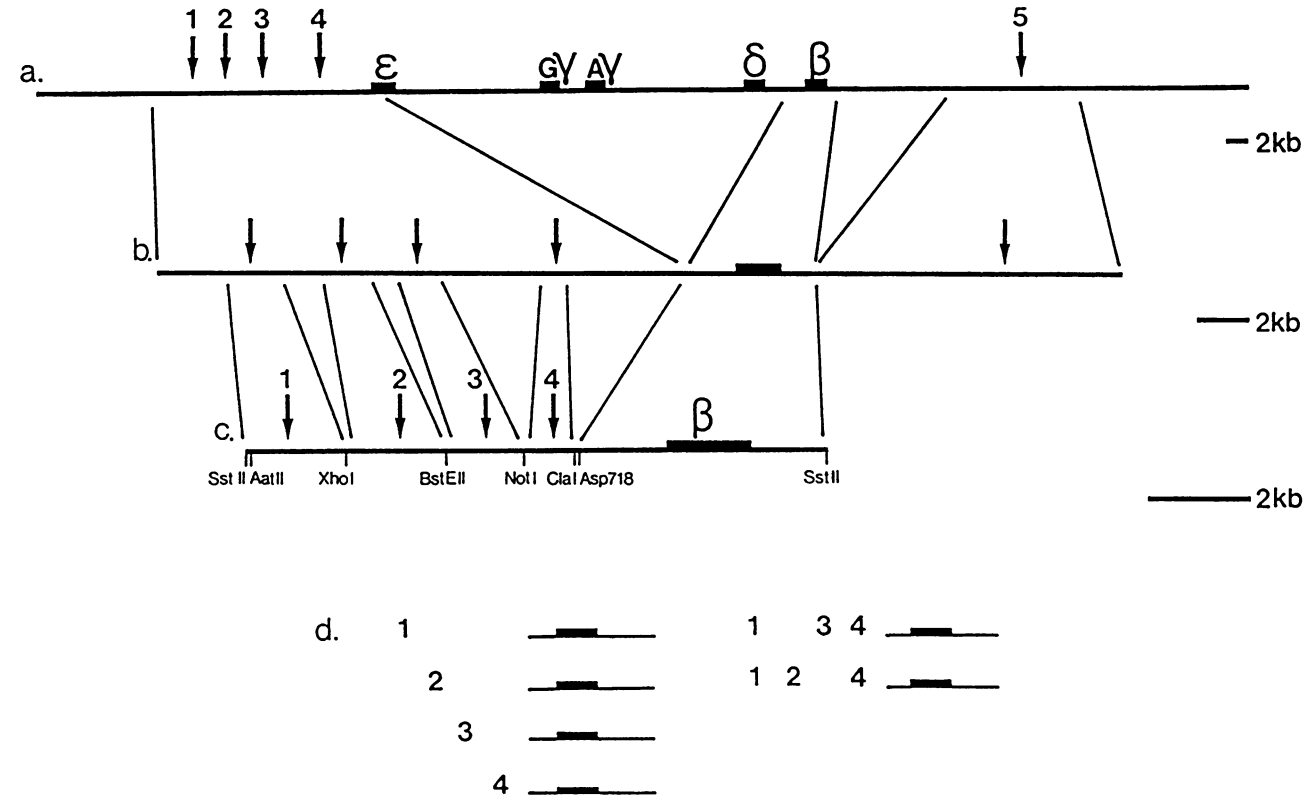

Fig. 1: Schematic illustration of the β-globin DCR constructs. (a) The human β-globin locus on chromosome 11. (b) The original cosmid β-globin minilocus as described in Grosveld et al. (15) was derived using the 5' and 3' elements illustrated. (c) The β-globin microlocus cassette containing the four DNaseI hypersensitive site fragments described in Talbot et al. (18). See Materials and Methods for detailed construction. The unique restriction enzyme sites flanking each hypersensitive site are illustrated. (d) Deletion variants of the microlocus micro-injected into fertilized mouse eggs to produce transgenic mice. Numbers represent the hypersensitive site(s) present in each construct.

1, 2 and 3 of the DCR provide position independent, copy number dependent expression and that both sites 2 and 3 are required for full expression of the human β-globin gene in transgenic mice.

MATERIALS AND METHODS

Constructs

Construction of the human β-globin microlocus cassette and microlocus deletion mutants was previously described by Collis et al. (24). Plasmid constructs were digested with SstII and purified from vector sequences on agarose gels.

Transgenic Mice

Purified fragments were microinjected into the pronuclei of fertilized mouse eggs and implanted into the oviducts of pseudopregnant (CBA×C57 Bl) F1 female mice (26). Foster mothers were sacrificed at 13.5 days of gestation and embryos were collected. Embryos were screened for the presence of the injected fragment via PCR and/or Southern blotting of placental DNA. Each transgenic was screened for mosaicism by Southern blotting of placenta, head and body DNA. Copy numbers were determined from dilution and Southern blotting of selected transgenic samples as described in the text, and by laser densitometry of Southern blots of placental DNA using a range of autoradiographic exposures.

Preparation of RNA

Frozen fetal livers were homogenized in 1.5 ml of 6M urea, 3M LiCl for approximately 60 seconds and sonicated for 1 minute. RNA was allowed to precipitate overnight at 4°C and collected by centrifugation at 10g for 30 minutes at 4°C (27). Pellets were washed once in the same solution, dissolved in 10mM Tris HCl (pH 7.5), 0.5% SDS, phenol-chloroform extracted and ethanol precipitated.

S1 nuclease protection assay

Globin RNA was analyzed by S1 nuclease protection assay (28,29). Probes were end labelled with T4 polynucleotide kinase. Twenty to thirty nanograms of labelled probe was hybridized to approximately 5μg of total fetal liver RNA in a total of 15μl of 40mM Pipes (pH 6.4), 400mM NaCl, 1mM EDTA and 80% formamide for 16 hours at 53°C. Samples were digested for 2 hours at 25°C with 100 u of S1 nuclease (Boehringer) in 250μl of 200mM NaCl, 30mM NaOAc (pH 4.5), 2mM $ZnSO_4$. The protected DNA was phenol-chloroform extracted, ethanol precipitated and electrophoresed on 6% urea-polyacrylamide gels. Individual bands were excised from the gel after autoradiography and Cerenkov counted. Probe specific activity was corrected by normalizing the ratio of human β-globin RNA to mouse globin RNA in Hu11 MEL cells to 0.5.

RESULTS

Generation of transgenic mice containing β-globin DCR deletion constructs

The original β-globin DCR construct consisted of 21kb of DNA encompassing the four erythroid-specific DNaseI hypersensitive sites located upstream of the ε-globin gene (15) (Fig. 1a,b). This was subsequently reduced to a 6.5kb cassette construct in which DNA fragments containing the individual hypersensitive sites were cloned into a synthetic polylinker and flanked by unique restriction sites (18,24) (Fig. 1c). Various deletion mutants were then produced by removal of one or three of the sites (Fig. 1d). Plasmid constructs were restricted with SstII and purified from vector sequences on agarose gels. DNA fragments were microinjected at 1−2 μg/ml into the pronucleus of fertilized mouse eggs and re-implanted into pseudopregnant females. Fetuses were collected at 13.5 days of gestation, DNA was prepared from individual placentas and screened for the presence of the transgene by polymerase chain reaction (PCR) (30) and/or Southern blotting (31).

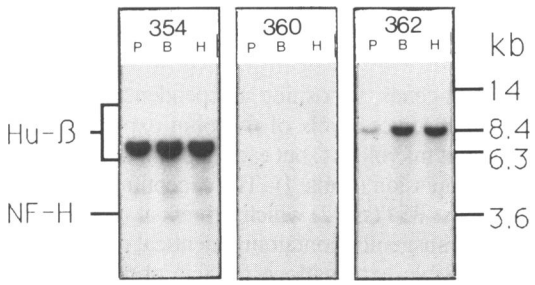

Fig. 2: Mosaic screening. Transgenic fetuses were screened for mosaicism by comparing the β-globin copy number from three different tissues. DNA from placenta (P), body (B) and head (H)was digested with BglII and Southern blotted. Blots were hybridized with Hu-β and NF-H probes as described in the text. This figure illustrates the three types of transgenic fetuses typically observed. Fully transgenic fetus (354); mosaic transgenic fetuses, placenta copy number higher than body and head (360), placenta copy numbers lower than body and head (362).

Mosaic Screening

Mosaicism is a common and misleading problem in analyzing expression levels in first generation transgenic fetuses (or mice). Accurate quantitation of expression levels per gene copy relies heavily on each cell of an expressing tissue having the same copy number. It is therefore critical that every possible step be taken to identify and exclude mosaic mice from the final analysis. We have found that the most reliable (though not completely infallible) method of identifying mosaic fetuses is by comparing the transgene copy number among at least three different tissues. Fetuses positive for placental DNA were therefore further analyzed to distinguish true transgenics from mosaic mice. DNA was prepared from head and body from each transgenic fetus, digested with BglII and Southern blotted alongside placental DNA. The blots were hybridized with a BamHI/ EcoRI probe from IVS II of the human β-globin gene and the single copy mouse neurofilament heavy chain (NF-H) cDNA as a loading control. Fetuses in which the transgene copy number varied between tissues were considered to be mosaic (Fig. 2).

Copy Number Determination

The copy number of non-mosaic transgenic fetuses was determined by Southern blot analysis of placental DNA as described above. The β-globin and NF-H specific bands in each lane of the autoradiograms were scanned with a laser densitometer and the β-globin signal was normalized with the value obtained

TABLE 1: EXPRESSION OF μ-LOCUS DELETION CONSTRUCTS IN TRANSGENIC MICE

Construct	Tg no.	cpm Huβ	cpm Mβ	Huβ/Mβ	Copy no.	Huβ/Mβ/ Copy no.
HS:1	140	1189	1751	0.68	5	0.14
	163	1471	1466	1.0	6−8	0.15
	174	119	266	0.45	20−25	0.02
HS:2	49	1166	1688	0.69	2	0.34
	77	2596	2674	0.97	3	0.32
	348	1160	1252	1.32	4	0.33
	354	1883	790	2.38	40−50	0.05
HS:3	91	458	1666	0.27	2	0.14
	104	2542	4103	0.62	4	0.16
	94	5344	7128	0.75	5	0.14
	103	8204	3974	2	12−18	0.13
HS:4	208	15	410	0.04	1	0.04
	198	27	833	0.033	2	0.02
	333	223	466	0.48	7	0.06
	335	877	576	1.5	30−40	0.04
	210	1222	668	1.8	>60	0.03
HS:124	188	79	99	0.26	3	0.26
	330	1347	1355	0.99	5	0.25
HS:134	32	100	293	0.34	1	0.34
	29	99	47	2.14	7	0.30
CONTROLS						
HS:1234	55	1377	922	1.49	3	0.49
Hu 11	MEL	271	543	0.5	1	0.50

TABLE I: Expression ratios of human β-globin RNA to mouse β-globin RNA. Individual bands from the S1 gel shown in Fig. 3 were excised and Cerenkov counted. The counts were corrected for background and adjusted for specific activity to give a ratio of Huβ/Mβ of 0.5 for Hu11 RNA (single human β-globin gene). Copy numbers were determined as described in the text.

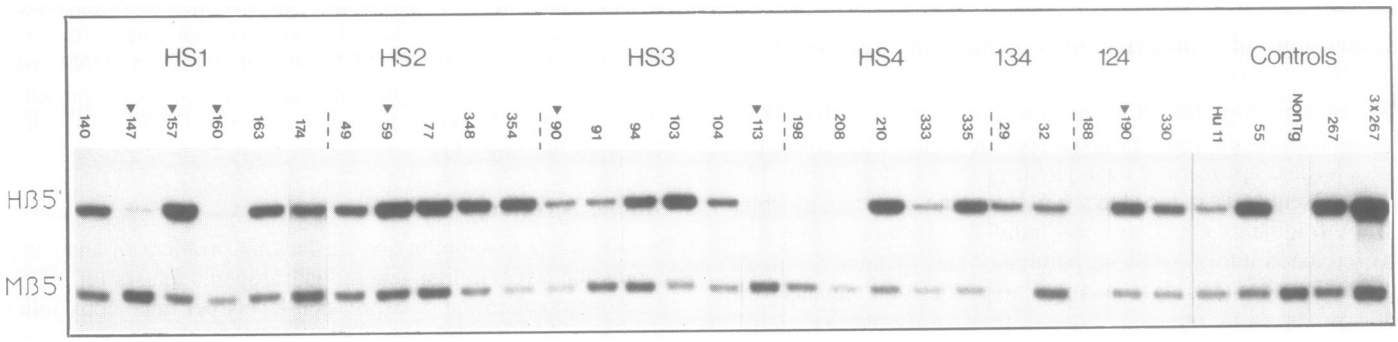

Fig. 3: Quantitative S1 nuclease protection assay of β-globin DCR deletion mutants. 5μg of fetal liver RNA from transgenic mice at 13.5 days of gestation was hybridized to 20−30ng of end-labelled DNA probe and treated with S1 nuclease. The 5′ human β-globin S1 probe yields a protected fragment of 160 nucleotides (Hβ5′); the 5′ mouse β-major globin protected fragment is 90 nucleotides (Mβ5′). Constructs are indicated HS1, HS2, etc., closed triangles indicate mosaic mice. Controls are, Hu11 RNA, 55 (fetal liver RNA from transgenic mouse with 3 copies of full μ locus), Non Tg, non transgenic mouse fetal liver RNA, 267 and 3× 267, 5μg and 15μg of transgenic fetal liver RNA to show probe excess.

from the NF-H specific band to obtain a relative copy number. These were arranged in a hierarchy from low to high transgene copy number and five DNA samples representing this range were selected. The copy numbers of these samples were then determined by a series of dilutions made with non-transgenic DNA to approximate the level of a single copy integrant. The signal obtained from the Southern blots of the diluted samples was compared to that of the single copy transgenic number 32 (based on end fragment analysis) and copy numbers were assigned. These values were then used as a scale in the estimation of copy numbers of transgenic fetuses in the hierarchy. Copy numbers ranged from single copy integrants to greater than 60 copies (Table I).

Expression Analysis of the DCR Deletion Mutants

Total RNA was isolated from 13.5 day transgenic fetal livers and analyzed by quantitative S1 nuclease protection assay with mixed probes for 5′ human β-globin and 5′ mouse β^{maj} globin mRNAs (Fig. 3). Total RNA from Hu11 MEL cells (32) and 13.5 day fetal liver RNA from a transgenic mouse containing three copies of the full microlocus (Fig. 1c) were used as controls (18). After autoradiography, individual bands were excised from the gel and Cerenkov counted in a scintillation counter. The expression of human β-globin was calculated as a ratio of the endogenous mouse β^{maj} globin and normalized per gene copy of the exogenous gene (Table 1).

Single Site Constructs

Transgenic mice containing single hypersensitive site constructs express significantly higher levels of β-globin RNA than those which contain no DCR hypersensitive sites (Fig. 3 and Table I). Site 2 expresses highest at nearly 70% of the level of a single endogenous β^{maj} globin gene. Site 1 and site 3 confer approximately 30% of the level. Site 4 transgenics express the lowest levels of human β-globin of the single hypersensitive site constructs at 4−10%. These results differ from those obtained in stably transformed MEL cell populations in which site 2 and site 3 provide 60 and 40% respectively, and site 1 and site 4 showed low levels of expression (<10%, 24). Clearly, in transgenic mice, site 1 is as capable as site 3 in producing high levels of β-globin RNA.

A distinctive feature of the minilocus (Fig. 1b) and microlocus (Fig. 1c), aside from providing high level expression of β-globin and other linked genes, is position independence. The single site constructs direct lower levels of β-globin expression per gene copy than the full microlocus, but each appears to provide position independent expression (Table I). The exceptions are mouse 174 (site 1) and mouse 354 (site 2) which express at much lower levels per copy than transgenics containing identical constructs. There are several possible explanations for this observation. Firstly, they could represent mosaics which have not been detected by our screening procedure. An equally likely explanation is that the reduced expression per copy in these transgenics is due to extremely high copy numbers of 20−25 and 40−50 copies respectively. Similar results were obtained in transgenic mice with high copies of β-globin DCR constructs containing site 3 (25) and site 2 (Philipsen et al., in press) and transfected MEL cells (Talbot et al., unpubl.). Clearly, the remaining single site transgenics with the possible exception of the site 4 transgenics, provide reproducible levels of expression per gene copy and thus we conclude confer position independence.

Three Site Constructs

Constructs 124 and 134 in which individual sites 3 or 2 have been deleted express human β-globin at lower levels than seen with the full microlocus (Fig. 3 and Table 1). Neither is significantly different from the level obtained with site 2 alone. This shows that sites 2 and 3 are required for full activity, and suggests that all four sites may be required. In conclusion, none of the constructs tested in transgenics which contain either single sites or combinations of three sites provide the full expression of the β-globin gene observed with the complete DCR.

DISCUSSION

We have previously shown that the full β-globin minilocus and microlocus provide position independent, high level expression of human β-globin in transgenic mice (15,18). The results presented here demonstrate that the function of position independence and copy number dependence can be provided by the individual sites 1, 2 and 3, and shows that sites 2 and 3 are required for full expression. This suggests that multiple sites act synergistically and are needed for the expression of each individual gene. Consequently, the presence of a number of DCR elements is not related to the fact that it is a multigene family per se.

Our results in the analysis of single site constructs differ from those obtained in stably transformed MEL cell populations (24) and transient CAT assays in K562 cells (22) or MEL cells (C-H. Chang and P. Dierks, pers. comm.). In transient assays, only site 3 was found to have significant enhancing activity. The results from stable populations of MEL cells showed that sites 2 and 3 had the strongest activating function. In transgenic mice we show that site 1 has similar transcriptional activating capacity as site 3 and that site 2 alone produces the highest levels of expression, approximately two-fold greater than 1 or 3 alone. The fact that sites 1, 2 and 3 are each capable of conferring position independent, copy number dependent expression is in apparent contrast to the interpretation of other laboratories which have studied the expression of β-globin-DCR hypersensitive site 3 constructs (site II in their nomenclature) in transgenic mice (20,25). Those studies however, did not exclude the presence of mosaic mice. The presence of such mice in those analyses is further suggested by the use of transgenic fetuses at 16 days, which is relatively late in the adult globin expression stage, and will actually select for mosaics. Fully transgenic mice which express high levels of human β-globin do not survive if expression is not balanced by α-globin expression (18,19). This probably explains why other laboratories have failed to find strict copy number dependent expression.

The expression level of human β-globin in transgenic fetuses with high transgene copy numbers appears to occur in a non-copy number dependent manner. This is particularly obvious in fetuses 174 (site 1; 20–25 copies) and 354 (site 2; 40–50 copies). This phenomena was also observed by Curtin et al. (25) in site 3 transgenic fetuses with copy numbers greater than fourteen and in MEL cells containing high copy number transgenes (Talbot et al., unpubl.). We feel this may be due to physical limitations created by tandem integration of several small DCR constructs. High copy number transgenic fetuses which contain the full minilocus, which retains the normal spacing of the hypersensitive sites, still express β-globin in a copy number dependent manner suggesting that trans-acting factors are not limiting (15). A more likely explanation is that the juxtaposition of several small DCR constructs creates an unusual chromatin structure which could extend for hundreds of kilobases, depending on the copy number. It is possible that such an artificial array of hypersensitive sites no longer allows each globin gene to be transcribed optimally.

An unexpected result from these experiments was the finding that at least the hypersensitive sites 1, 2 and 3 taken individually direct position independent expression from a linked globin gene. This apparent redundancy suggests that the sites may operate independently or have varied functional activities which are developmentally stage specific. This is suggested by the difference in activity found for site 1 constructs when assayed by transfection in adult type MEL cells (24) and the transgenic fetal liver. Mice containing a site 4 construct are active but only express at low levels and it is not clear whether it is independent of position of integration. This is in contrast to the *in vivo* results obtained in a patient with γδβ-thalassemia containing site 4 only in which the β-globin allele *in cis* is completely silent (33). These results suggest, therefore, that site 4 may not play an important role in adult globin expression in the context of the entire locus. However, its activity may be increased significantly in earlier developmental stages in conjunction with ε- or γ-globin gene expression. Likewise, sites 1, 2 and 3 may have different specificities for different genes at each developmental stage.

One of the aims of this work is to increase the understanding of the mechanisms underlying globin-DCR interactions and thus delineate the minimal requirements for efficient expression for subsequent use in somatic gene therapy constructs/experiments. Our data shows that at least sites 1, 2 and 3 should be present and we are presently reducing the size of the individual hypersensitive sites to minimal core fragments which retain full activity of the larger fragments presented here (Talbot et al., in press, Philipsen et al., in press, Hanscombe et al., unpubl.). With this information we will develop smaller β-globin DCR constructs suitable for insertion into retroviral vectors and use in gene addition experiments and gene therapy.

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