Tissue-Specific and Ectopic Expression of Genes Introduced into Transgenic Mice by Retroviruses

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Recombinant retroviruses containing the complete genomic human β globin gene (under the control of its own promoter) and the bacterial neomycin phosphotransferase gene (under the control of the normal or enhancerless viral promoter) were used to derive transgenic mouse strains by infection of preimplantation embryos. Expression of the β globin gene in hematopoietic tissues was observed in all transgenic strains. In addition, one strain showed ectopic expression of β globin in the same tissues that also expressed high levels of RNA from the viral promoter. It is likely that expression from the long terminal repeat (LTR), in contrast to expression from the internal promoter, is dependent on the site of integration. Thus, retroviral vectors can be used for tissue-specific expression of foreign genes in transgenic mice, as well as for the identification of loci that allow developmental activation of a provirus.

THE INTRODUCTION OF FOREIGN genes into the germ line of mice has made it possible for investigators to study the mechanisms of gene activation during mouse development and the effect of altered gene expression on the animal. Microinjection of DNA into the zygote pronucleus has been highly successful in generating transgenic mice that express the inserted gene in a tissue-specific manner (*1*) resulting in phenotypic rescue of a mutant phenotype in some instances (*2*). An alternative method for insertion of foreign genes into animals is based on infection with retroviruses; this approach is not restricted only to the one-cell embryo but can be used throughout prenatal and postnatal life to introduce foreign genes into the germ line (*3*) and into hematopoietic stem cells (*4*). For these reasons, the retrovirus has been useful as an insertional mutagen or as a chromosomal marker for the X/Y chromosome pairing region (*5*), and as a lineage marker for early mouse development (*3*) as well as for later stages of cell differentiation (*4*).

The usefulness of retroviral vectors for gene transfer into animals has been severely hampered by the lack of provirus expression once introduced into the early embryo or into embryonic stem cells (*6*). This block in expression is maintained throughout later stages of development and postnatal life (*7*) by a *cis*-acting mechanism that probably involves methylation of the proviral genome (*8*). It appeared possible, however, that a gene transcribed from an internal promoter (*9, 10*) might be regulated independently of the inhibitory effect of the proviral long terminal repeat (LTR); transfer of such a gene might result in developmentally regulated gene activation. We therefore introduced retroviral vectors containing a genomic human β globin gene under the control of its own promoter, as well as a neomycin phosphotransferase (*neo*) gene under the control of the viral LTR into the mouse germ line. These vectors had been shown previously to result in regulated expression of the β globin gene when introduced into murine erythroleukemia (MEL) cells (*10*). We now show that the β globin gene is expressed in a tissue-specific pattern in three transgenic mouse lines, and that, unexpectedly, the LTR-driven *neo* gene is strongly expressed in muscle tissues of one mouse line.

The two retroviral constructs that we have used to infect embryos are shown in Fig. 1A (*10*). The titer of the cell line producing virus with an intact LTR (en^+) was 2×10^5 G418-resistant colony-forming units per milliliter. The titer of the cell line producing virus with a deletion of the viral enhancers (en^-) was estimated by infecting 3T3 cells and assaying for the presence of proviral copies by blot hybridization, since removal of enhancer sequences leads to a considerable reduction in expression of *neo*. The titers of the two producer cell lines were similar when assayed by this procedure. Similar types of vectors to the en^- vector have been described recently (*11*). CFW preimplantation mouse embryos were infected with the two viral constructs and founder mice were derived as described (*3*). By means of Southern blot analyses of DNA's from tails, three transgenic mouse lines carrying the en^+ virus (Glob 1) or the en^- virus (Glob 2 and Glob 3) were derived from a total of 69 mice that had been exposed to virus as embryos. This efficiency of embryo infection (about 5%) is low in

Table 1. Human β globin and viral transcripts in transgenic mice. RNA transcripts were quantitated by Cerenkov counting of bands cut out from the dried gels after autoradiography or by means of an LKB laser densitometer; this was compared with the level of transcripts detected in human blood or in ψ2 cell lines producing the LTR enhancer-plus virus with a titer of 2.10^5 G418-resistant colony-forming units per milliliter (*10*). Ribonuclease protection experiments with a mouse β globin SP6 probe show that the level of endogenous β globin expression is similar in mouse or human blood (data not shown). Under the assumption that 1% of total cellular RNA is mRNA, the fraction of β globin or viral RNA present was calculated by correlating the number of counts in a band with dilutions of known amounts of unprotected probe, taking into account the full length of the transcript, the length of the protected fragment, and the specific activity of the probe. These calculations were based on the assumption that 100% efficiency of hybridization is reached during annealing, and therefore provide a lower estimate. The level of β globin transcripts in human blood and of viral transcripts in the producer cell line was estimated to be 6% and 0.44% of mRNA, respectively. In the measurements of viral transcripts, quantitation was also based on densitometry of Northern blots. +, Expression detected but not quantitated; −, no expression detected; ND, not determined.

Animal	Tissue	Human β globin (% of level in human blood)	Viral transcripts (% of level in producer line)
Glob 1-1	Blood	+	−
	Heart	+	55
Glob 1-2	Blood	0.08	−
	Heart	0.04	+
	Skeletal muscle	0.03	18
	Smooth muscle	+	6.6
Glob 1-3	Blood	−	−
	Skeletal muscle	0.005	2.2
Glob 1-4	Blood	ND	ND
	Skeletal muscle	ND	13
	Smooth muscle	ND	2.8
Glob 2-1	Blood	0.13	−
Glob 2-2	Blood	0.11	−
Glob 3	Blood	0.01	−

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comparison to the efficiency of more than 50% that is obtained with Moloney murine leukemia virus (M-MuLV) (*3*) and might be attributed to a 25-fold difference in titer between the cell lines that were used. A difference of about 100 nucleotides in the respective proviral LTR's as seen in blot hybridizations was used to confirm the presence or the absence of the M-MuLV enhancer in the Glob strains (Fig. 1B). Restriction mapping with several different enzymes identified unique cellular flanking sequences, demonstrating that only one provirus was carried in each mouse strain (Fig. 1B).

To study lineage-specific globin expression, Glob-strain mice were treated with phenylhydrazine to induce erythroid anemia, which results in an increase in the number of reticulocytes. Human β globin–specific transcripts were detected by ribonuclease (RNase) protection mapping by means of antisense RNA's synthesized from an SP6 promoter (*10, 12*), which maps accurate transcription initiation from the β globin promoter. In two Glob 1 animals, transcription of the globin gene was detected in the blood, bone marrow, and spleen (Fig. 2 and Table 1). The third Glob 1 animal expressed lower amounts of the human β globin messenger RNA (mRNA), which was only detected after a longer exposure of the gels. In addition, all three animals exhibited variable expression in nonhematopoietic tissues, especially in the heart, skeletal muscle, and smooth muscle; in two animals (Glob 1-1 and 1-3), expression was higher in nonhematopoietic tissues than in the blood. In the Glob 2 and the Glob 3 animals, human β globin transcripts were mainly detected in the blood, suggesting that the pattern of expression of the foreign β globin gene in these strains was more specific for hematopoietic tissues. In all three strains, faint expression in other organs such as the lung and the thymus was probably due to contamination of the tissues with blood. Similar levels of human β globin RNA were detected in the blood of the Glob 1 and the Glob 2 strains, whereas the Glob 3 strain expressed less RNA (Table 1).

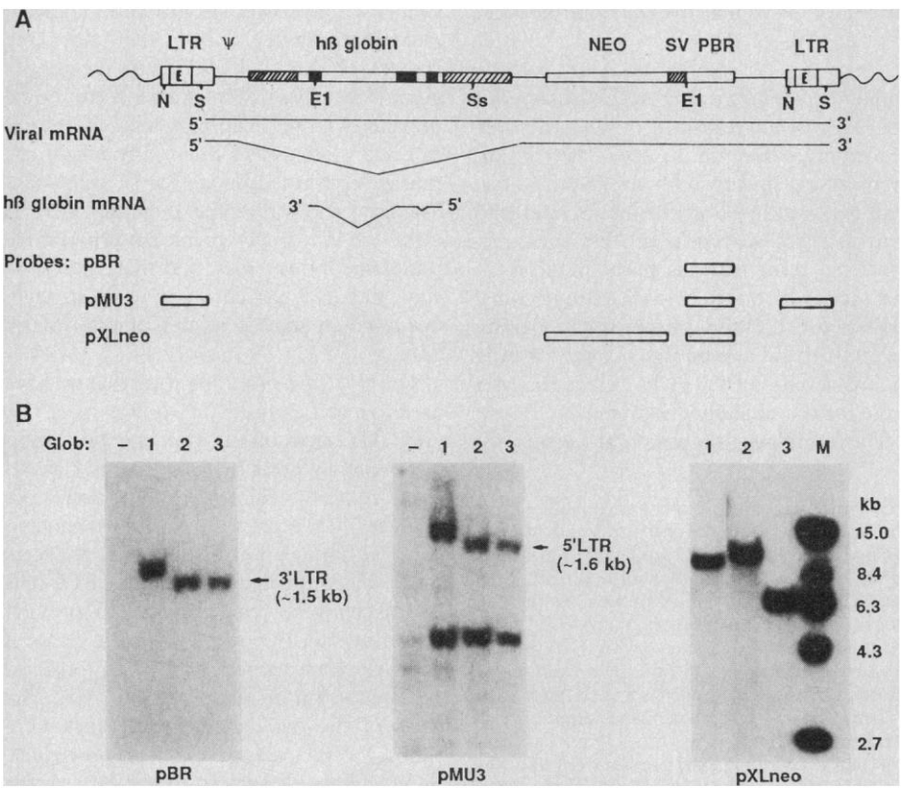

Fig. 1. Structure of proviral genomes. (A) Structure of retroviral vectors. A 3-kb human (h) β globin sequence, extending from a Hpa I site 815 nucleotides upstream of the mRNA cap site to a Pst I site 2.2 kb downstream from this site, was inserted in the reverse orientation into the retroviral vector pZip-neoSV(X) (*19*) or pZip-neoSV(X) en$^-$ (*10*) and introduced by transfection into ψ2 cells. A deletion of the 3′ LTR enhancer (E) in the en$^-$ constructs was made from a Pvu II site at M-MuLV nucleotide 7933 (*20*) to an Xba I site at nucleotide 8111 and is transferred to the 5′ LTR upon integration of the provirus. ψ, the psi sequence necessary for encapsidation of retroviral genomes; hβ globin: hatched boxes, flanking cellular sequences; open boxes, noncoding 3′ and 5′ sequences, and intervening sequences; black boxes, coding sequences. Inserts corresponding to the *neo* gene (NEO), the SV40 (SV) and pBR322 (PBR) origins of replication have been described previously (*19*). N, S, Ss, and E1 underneath the boxes represent cleavage sites for Nhe I, Sac I, Ssp I, and Eco RI, respectively. Sequences hybridizing to pBR322 (pBR), pMU3, and pXLneo are indicated; pMU3 is a plasmid containing a head to tail dimer of an Rsa I fragment (nucleotides 7762–8297) from the LTR inserted in pUC9; pXLneo contains the origin of replication of pBR322 and the *neo* gene (*10*). (B) Provirus structure in the Glob 1, 2, and 3 strains. Procedures for DNA purification and blot hybridization have been described previously (*3*). DNA's isolated from tails of control (–) or Glob animals (1 to 3) were digested with Sac I and Eco RI, with Nhe I and Eco RI, or with Ssp I, and hybridized with pBR322, pMU3, or pXLneo, respectively. Hybridization with pBR322 reveals sequences specific for 3′ LTR. Hybridization with pMU3 reveals the 5′ LTR (top band), an internal segment (bottom band), and single 3′ cellular flanking sequences (not shown); weak bands in control animals are due to cross-hybridization of the probe with endogenous retroviral genomes. Hybridization with pXLneo demonstrates the presence of single proviral insertions in each strain. Additional Ssp I sites at the 5′ end of the provirus have been omitted from the map.

After phenylhydrazine treatment, the spleen becomes a major site of erythropoiesis. The three strains expressed human β globin in the spleen, suggesting that the expression of the inserted gene may have been erythroid lineage-specific. This result was obtained irrespective of whether the provirus did or did not carry the enhancer in the LTR, as was found with MEL cells infected with both virus constructs (*10*). Thus, expression of genes under the control of internal promoters may be independent of the viral LTR. Furthermore, since this pattern of expression was observed in three different strains, tissue specificity of expression appears to be independent of the site of proviral integration.

The level of human β globin expression in all mice was much lower than the level of expression of the mouse β globin gene, or of β globin found in human blood. This may be due to the presence of prokaryotic sequences in the retroviral vector, which have been shown to strongly inhibit expression of the β globin or the α fetoprotein (*13, 14*) genes in transgenic mice. Alternatively, low expression may have resulted from integration at sites in the cellular genome that would not be permissive for high levels of expression of the β globin gene. The different amounts of human β globin mRNA in the three transgenic strains indeed suggest that the level of transcription might be influenced by the site of integration. In addition, our virus construct may lack the 3′ enhancer-like sequences that have been shown recently to be important for efficient β globin expression (*15*).

One puzzling feature of the Glob 1 strain is the variability of gene expression from one animal to another. Variability of expression has also been observed in transgenic mouse strains produced by microinjection into zygotes (*16*). In this case, the pattern of gene methylation acquired by the original

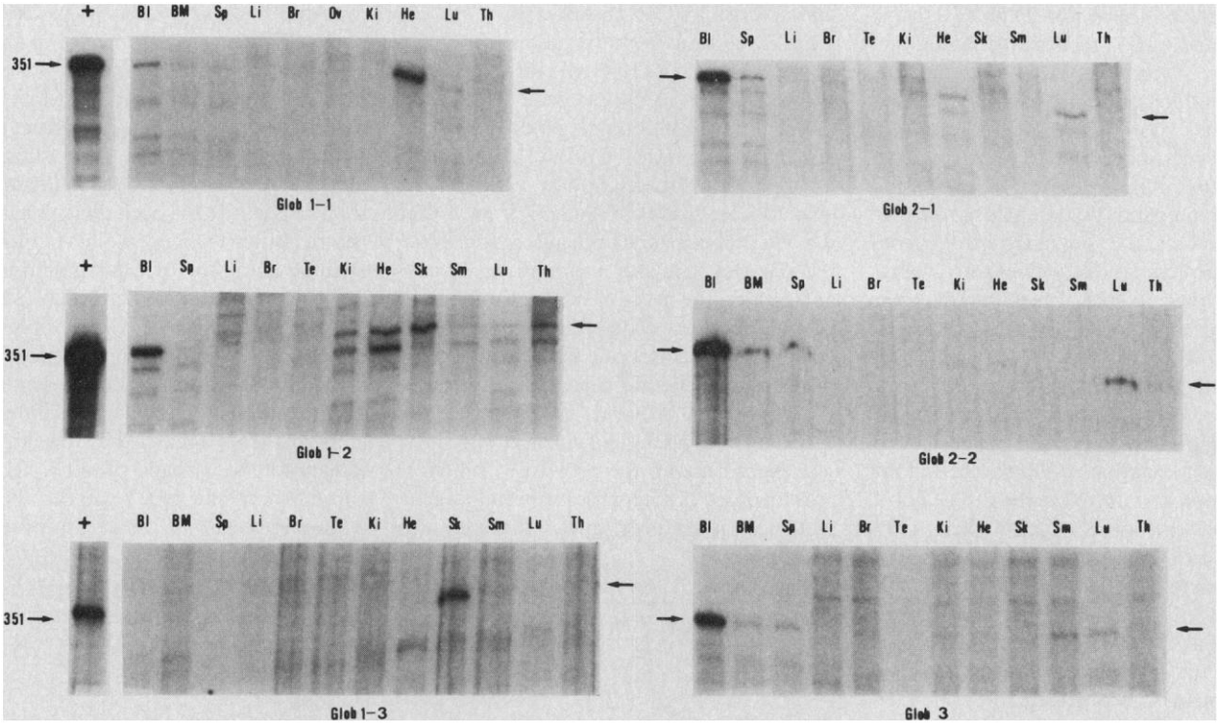

Fig. 2. Expression of human β globin in transgenic animals. RNA's from blood (Bl), bone marrow (BM), spleen (Sp), liver (Li), brain (Br), ovaries (Ov), testis (Te), kidney (Ki), heart (He), skeletal muscle (isolated from the legs; Sk), smooth muscle (stomach; Sm), lungs (Lu), and thymus (Th) were purified as described (*7*). +, RNA isolated from human blood; −, yeast transfer RNA. Total cellular RNA's (10 μg) from three Glob 1 animals (1–3), two Glob 2 animals (1 and 2), and one Glob 3 animal were hybridized with an excess of [^{32}P]-labeled complementary human β globin SP6 probe as described (*10*) except that hybridizations were carried out at 55°. After RNase digestion, the protected products were denatured and separated on a 6% polyacrylamide denaturing gel. A protected fragment of 351 bases indicates accurate transcription of the human β globin gene. Because the gels did not always run evenly, the position of this protected fragment is indicated by arrows. The double-protected bands seen with kidney and heart of animal Glob 1-2 represent a loading artifact. Different exposure times (3–15 hours) are shown for different animals, and underexposures of the film are shown for lanes containing human blood RNA (+).

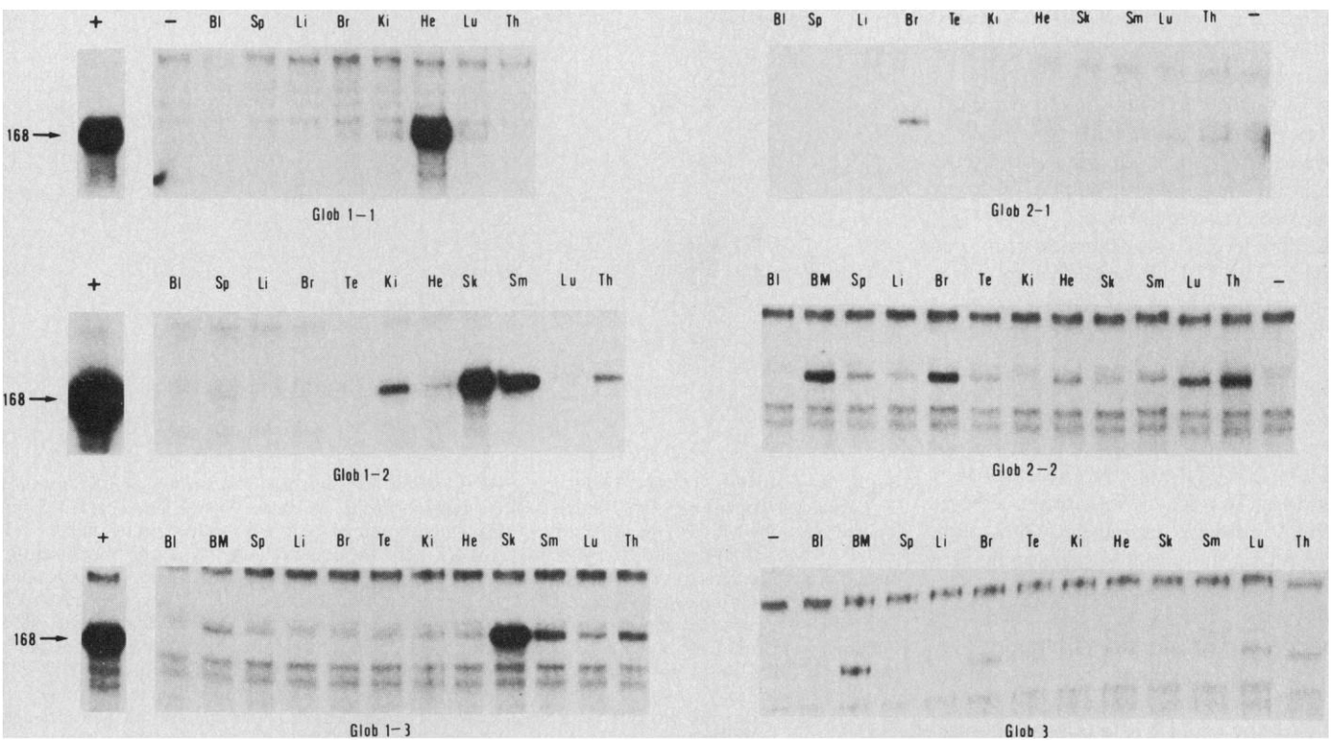

Fig. 3. Expression of *neo* in transgenic animals. Experimental procedures were as described in the legend to Fig. 2 except that + refers to RNA isolated from the LTR enhancer-plus producer cell line. A protected fragment of 168 bases is specific for *neo*.

founder animals was not inherited by all offspring and was correlated with changes in expression. Preliminary evidence suggests that proviral genomes are more methylated in Mov mice (*8*) than in Glob 1 mice. We have, however, no evidence for changes in methylation of the provirus in expressing and nonexpressing tissues. Moreover, we cannot exclude the possibility that genes affecting expression of the viral insert segregated in the Glob 1 mice, which were established in an outbred strain, and contributed to the variable gene activity.

To study whether *neo* was expressed, RNase protection experiments were performed (Fig. 3) with an SP6 probe specific for the 3′ end of *neo* (*10*). High levels of *neo* expression were detected in the three Glob 1 animals in the same organs where nonhematopoietic expression of the globin gene had been detected (Table 1). No *neo* expression was seen in the blood of these animals. In the Glob 2 and Glob 3 animals, which contained an en^- provirus, a very low level of *neo* expression was detected in bone marrow, brain, lung, and thymus. In Glob 1 mice, Northern blot analyses of RNA from muscle tissues revealed two transcripts of the same size as detected in the ψ2 producer cell lines, indicating that the viral transcripts were correctly spliced (Fig. 4A). The same result was obtained with animals that had not been treated with phenylhydrazine, ruling out the possibility that this treatment induces expression of *neo*. RNase mapping with a M-MuLV LTR SP6 probe (Fig. 4B) indicated that transcription was initiated from the M-MuLV promoter. The level of transcripts in muscle was similar, in some cases, to that observed in the en^+ producer cell line (Table 1). So far, we have been unable to map transcription initiation in the en^- mice to the viral promoter by means of RNase protection analysis.

The high level of *neo* expression promoted by the M-MuLV LTR in Glob 1 mice was unexpected in light of previous experiments that failed to detect any transcriptional activity of the M-MuLV or MSV LTR's in transgenic strains (*7*) or in chimeras produced with infected embryonal stem cells (*17*). To confirm transcriptional inactivity of the M-MuLV LTR, different tissues of Mov 7 and Mov 10 mice (*3, 7*) carrying a single M-MuLV provirus in the germ line were analyzed with the SP6 RNase mapping procedure. No viral transcription was detected in Mov 7 or Mov 10 mice (Fig. 4C). The high rate of transcription from the viral LTR in tissues of Glob 1 mice is therefore exceptional and does not reflect the normal activity of the viral LTR in transgenic mice.

There are several possibilities to explain the strong *neo* expression detected in Glob 1 mice particularly in muscle tissues. (i) Ectopic expression of the β globin gene may activate the viral LTR in a similar manner as has been observed in transgenic mice carrying two adjacent foreign genes (*18*). This explanation appears unlikely because no LTR-promoted transcription was seen in hematopoietic tissues of Glob 1 mice that actively transcribe the human β globin gene. (ii) Without sequence information, we cannot exclude the possibility that the LTR in Glob 1 mice has acquired a mutation that promotes muscle-specific transcriptional activation. (iii) The most likely interpretation is that the viral genome is activated in the specific tissues because of an effect of cellular sequences flanking the provirus. The chromosomal site of integration of foreign gene sequences introduced into transgenic animals has been suggested previously to explain different developmental patterns of activation of M-MuLV proviruses in Mov mice (*3*) and ectopic expression of the human β globin gene (*13*). We have recently obtained direct evidence supporting such a mechanism of virus expression in embryonic stem cells (*6*). It has been difficult to validate this hypothesis when genes were introduced into the germ line by DNA microinjection, because this technique generally leads to the integration of multiple copies of the foreign sequences, often in large tandem arrays, making it difficult to clone the genomic site of integration. Infection of preimplantation embryos with retroviruses results in the integration of a single provirus at a given chromosomal site, which can then be cloned with relative ease (*3, 5*). Preliminary indica-

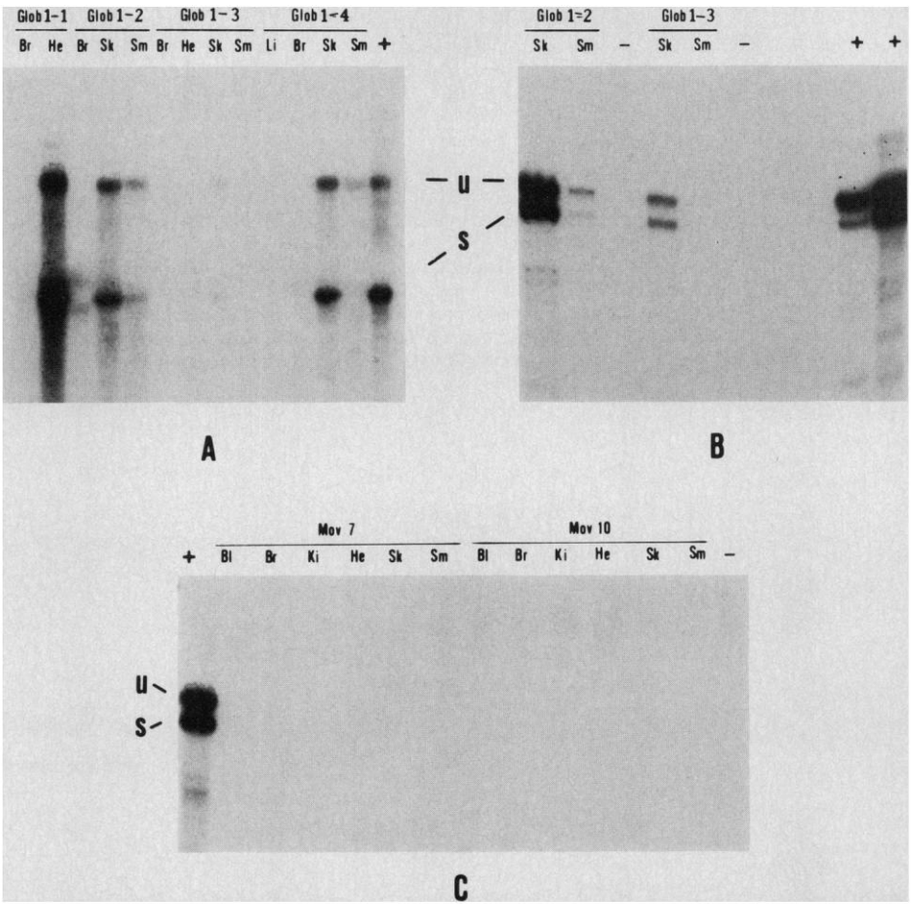

Fig. 4. Activity of the M-MuLV LTR in the Glob 1 strain. (A) Northern blot analysis of RNA's isolated from different organs of Glob 1 animals (see legend to Fig. 2). Animals Glob 1-1, 1-2, and 1-3 were treated with phenylhydrazine as described (*14*), whereas animal Glob 1-4 received no treatment. Total cellular RNA's from organs (10 μg) or from the LTR en^+ producer cell line (+; 2 μg) were denatured with glyoxal, separated on a 1% agarose gel, transferred to GeneScreen Plus membranes (New England Nuclear), and hybridized to pXLneo. U and S represent unspliced and spliced RNA's, respectively. (B) Ribonuclease protection analysis of RNA's isolated from organs of Glob 1 animals. Total cellular RNA (10 μg) from two Glob 1 animals were hybridized with SP6 complementary RNA's synthesized by means of pSPMLV (a gift of E. Barklis), which contains the Sac I–Bal I (nucleotides 8229–212) segment of M-MuLV) inserted in pSP64. Protected fragments of 214 and 205 bases represent accurate transcription from the M-MuLV promoter for the unspliced (U) and the spliced (S) messages, respectively; +, RNA's (1 μg) from two different producer cell lines. (C) Ribonuclease protection analysis of RNA's isolated from organs of Mov 7 and Mov 10 mice. Total cellular RNA (10 μg) was hybridized to the SPMLV probe; +, skeletal muscle of animal Glob 1-2.

tions of transcriptional activity of the host sequences flanking the provirus support the possibility that expression of the *neo* gene in Glob 1 mice may be due to the chromosomal position of the provirus. Globin expression in the nonhematopoietic tissues may in turn be due to activation of the internal promoter by the viral LTR.

The analysis of the three strains of transgenic mice presented in this report provides the first demonstration that retroviruses can be used for the introduction and tissue-specific expression of foreign genes into the mouse germ line. Our results indicate that a gene under the control of an internal promoter and transduced by a retroviral vector is responsive to *trans*-acting developmental signals, resulting in tissue-specific gene activation. Similar vector constructs expressing the gene of interest from an internal promoter may not only be appropriately expressed after transfer into mouse embryos, but may also function properly after introduction into stem cells of other lineages such as the hematopoietic system.

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Physical Theory of the Orientation of Astral Mitotic Spindles

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A physical theory was developed for mitotic spindle orientation. This is important because spindle position is known to determine the placement of the cleavage furrow separating the offspring of a mitosis. The theory is based on an equation for the force exerted on spindle poles by the interaction of astral microtubules with the cell surface. Expected spindle placements are positions of stable equilibrium where the net force and torque resulting from the action of the astral microtubules on the spindle poles is zero. The theory provides a novel physical explanation of cleavage patterns in the early embryo.

THE FIRST FEW MITOSES IN THE EARly embryo are often described as if they had crystalline perfection. "The plane of the first division is as a rule vertical; it passes through the main axis of the egg. The plane of the second division is also vertical and passes through the main axis, but it is at right angles to the first plane of cleavage. . . . The plane of the third division is at right angles to the first two planes and to the main axis of the egg. It is therefore horizontal or parallel to the equator of the egg" (*1*, p. 143). This is a textbook simplification; nonetheless it captures the essence of the pattern. Little is known about the physical basis of the orientation of mitosis in any cell (*2*). I describe a physical theory which, though simple, seems to capture the essence of the astral mitosis orientation process in general, including that which creates cleavage patterns.

The placement and orientation of the mitotic spindle determine the plane of cleavage in animal cells (*2, 3*). The problem of mitosis orientation may thus be profitably recast as the problem of spindle orientation. The spindle in anaphase may be viewed as a relatively rigid structure with an aster at each pole (Fig. 1). Asters are so named because the thousands (*4, 5*) of microtubules radiating from them give a starlike appearance (*2, 6, 7*). In anaphase the astral microtubules grow to reach the cell surface (*2, 5–7*). Hill and Kirschner (*8*) have proposed that the ability of a microtubule to exert a longitudinal force is due to polymerization of the tubule. This theory suggests that astral microtubules can exert a displacement force on the spindle pole, if they contact the cell surface or internal structures.

To calculate spindle position we must know the force exerted on the spindle poles by each astral microtubule. I assumed that the force per microtubule decreases with length squared. This relation was derived as follows. Hill and Kirschner (*8*) showed that the polymerization force has the form

$$-\frac{k_B T}{\ell_0} \ln \frac{C_e}{C_e^0} \sim 1 \times 10^{-6} \text{ dyne per tubule}$$

(k_B is Boltzmann's constant, T is temperature, ℓ_0 is the length of polymer per monomer, C_e is the concentration of free monomer at equilibrium, and C_e^0 is the critical concentration for polymerization). In their derivation, they assumed for simplicity that the tubules had no bending moment. This assumption is adequate for short tubules (<1 μm) or for situations where $C_e/C_e^0 \approx 1$. For long tubules, however, such as those found in the aster, buckling of the tubule would occur at forces weaker than the force available through polymerization (assuming no cross-linking of astral microtubules). The critical buckling force of a long thin rod of circular cross section is approximately $\pi^3 ER^4/(4L^2)$ where E is Young's modulus, R is the radius of the rod, and L is the length of the rod (*9*). Given that $E = 1.1 \times 10^8$ dyne cm^{-2} (*10*) and $R = 1.4 \times 10^{-6}$ cm, the critical buckling

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