should favor the use of transgenic mice as models of metastasis and neoplasia.


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20. We thank E. F. Aglow for histologic preparation and J. Weibel for electron microscopy. Supported by USPHS grants CA-10815, CA-18470, CA-21124, and CA-25875 from NCI and grants HD-17720, HD-23291, and HD-23239 from NICHD and a grant from the Swiss National Science Foundation. N.F. was a Leukemia Society of America Special Fellow and R.C. was supported by T32-CA-23176.

21 November 1988; accepted 17 February 1989


# Mouse Embryonic Stem Cells and Reporter Constructs to Detect Developmentally Regulated Genes


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**A strategy was devised for identifying regions of the mouse genome that are transcriptionally active in a temporally and spatially restricted manner during development. The approach is based on the introduction into embryonic stem cells of two types of *lacZ* reporter constructs that can be activated by flanking mouse genomic sequences. Embryonic stem cells containing the *lacZ* constructs were used to produce chimaeric mice. Developmental regulation of *lacZ* expression occurred at a high frequency. Molecular cloning of the flanking endogenous genes and introduction of these potential insertional mutations into the mouse germ line should provide an efficient means of identifying and mutating novel genes important for the control of mammalian development.**


TO GAIN A BETTER UNDERSTANDING of mammalian development, it is essential for one to identify genes and mutations involved in establishing the basic body plan around the time of gastrulation and early organogenesis. The experiments described here are aimed toward this goal, the rationale arising from our earlier results in which the integration of the *lacZ* gene linked to a weak promoter was shown to detect cis-acting elements in the mouse genome that activated β-galactosidase expression in the developing spinal cord and caused a neurological mutation (*1*). Similar developmental activation of *lacZ* constructs was reported in *Drosophila* (*2*) and other transgenic mouse strains (*3*). The use of transgenic mouse lines to detect developmentally regulated genes, however, is limited by the number of integration events that can be readily analyzed. Thus, we have developed a strategy in which mouse embryonic stem (ES) cells (*4*) and two different types of *lacZ* constructs are used to screen many integration events and to rapidly clone the associated genes. ES cells fully retain their pluripotent character after a variety of genetic manipulations in vitro and efficiently form chimaeras after reinjection into blastocysts (*5*). This property allowed us to introduce reporter gene constructs into ES cells and analyze their expression directly in chimaeric embryos without the necessity of generating transgenic mouse lines.

Two types of reporter constructs were used in these studies. In the first, which we term an "enhancer trap" construct (Fig. 1A), the *lacZ* gene was fused in frame to a minimal promoter derived from the mouse heat-shock protein 68 (*hsp68*) gene that provides a TATA box and translation initiation codon but is not sufficient on its own for expression of β-galactosidase in mouse ES cells (*6*). The construct also contained the bacterial *SUIII*$^+$ suppressor gene and the bacterial neomycin resistance (*neo*) gene. In this construct, expression of the *lacZ* gene should depend on cis-acting regulatory elements close to the site of integration that activate the weak *hsp68* promoter.

In contrast to fertilized eggs microinjected with DNA, ES cells permit selection for rare-occurring integration events, which allowed us also to design and use a "gene trap" vector (Fig. 1B). The vector contains

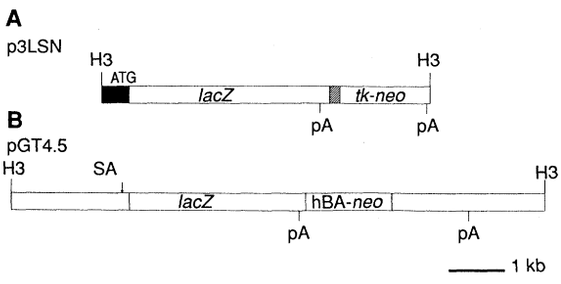


**Fig. 1.** *LacZ* gene constructs used as reporter genes. (**A**) The enhancer trap construct p3LSN: a 0.5-kbp Stu I–Nco I fragment of the mouse *hsp68* gene (filled-in box) was used as a minimal promoter (*15*). This fragment contains two heat-shock elements upstream of the TATA box and translation initiation codon. To avoid any possible regulatory effects we also made an additional construct, p6LSN, in which the heat-shock elements were deleted. No obvious difference was observed between the two in our studies. The promoter was linked in frame to *lacZ* (pMC1871; Pharmacia). The bacterial *SUIII*$^+$ gene (cross-hatched box) was obtained from piVX (*16*). The *neo* gene under the control of the herpes simplex virus *tk* promoter was taken from pMoTN (*17*). (**B**) The gene trap construct pGT4.5: mouse *En-2* genomic DNA (stippled box), consisting of a 5′ 1.8-kbp Eco RI–Sst I fragment including the homeobox-containing exon (*7*), was joined in frame to the *lacZ* gene. SA indicates the splice acceptor site of the homeobox exon and hBA-*neo* consists of the human β-actin promoter driving *neo* (*9*). A 3′ 2.7-kbp Bgl II–Sst I *En-2* genomic fragment containing the 3′ untranslated region provided the polyadenylation signal for *neo*. The Hind III site within the 5′ *En-2* intron sequences was deleted. Both constructs contain unique Hind III (H3) sites at their ends. Polyadenylation signals (pA) were derived from SV40.


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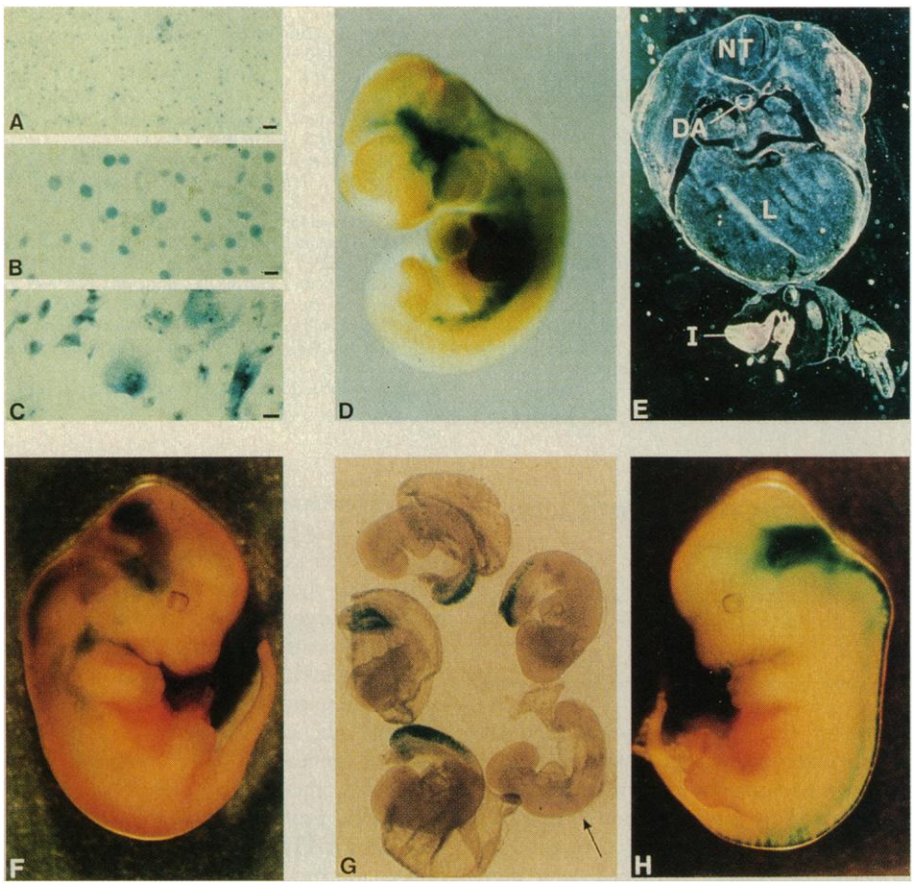


**Fig. 2.** Expression of *lacZ* in cells and chimaeric embryos. (A to C) Partially differentiated cell lines containing pGT4.5 on gelatinized plates. (**A**) GT-1 cells showed β-galactosidase activity concentrated in a single "dot" in the cytoplasm. (**B**) Nuclear localization of β-galactosidase activity in GT-2 cells. (**C**) Strong staining in the cytoplasm and within presumptive cytoplasmic vesicles of GT-10 cells. Bars represent 10 μm. (D and E) Expression of *lacZ* in GT-10 chimaeric embryos. In day 8.5 embryos the most obvious staining was in two ventrolateral stripes located beneath the somites. At 10.5 (**D**) and 12.5 days, staining was observed in two ventrolateral stripes at and below the surface of the embryo and in cells surrounding lumina of blood vessles, the intestine, and the otocyst. (**E**) Dark-field photomicrograph of a cross section through the midsection of a day 12.5 embryo showing this pattern; β-galactosidase staining appears pink. DA, dorsal aorta; NT, neural tube; I, intestine; and L, liver. (**F**) Day 12.5 chimaeric embryo from the D3-3-15 cell line. Expression of β-galactosidase was restricted to parts of the central nervous system and two ill-defined lateral stripes near the forelimb buds. The *lacZ*-expressing cells were first detected at day 9 in the developing myencephalon. (G and H) Staining of *lacZ* in D3-6-28 chimaeric embryos. No *lacZ* expression was observed in day 7.5 chimaeric embryos, whereas between day 8.5 and day 9 expression was confined to the posterior neural tube. (**G**) Five day 9 embryos obtained from one series of injections. Arrow indicates a presumed nonchimaeric embryo. By day 10.5 to 12.5 (**H**), expression extended along the entire length of the spinal cord and into the hind brain.

the *lacZ* gene, lacking a promoter and translation initiation signal, inserted in frame into the homeobox exon of the *En-2* gene (*7*) such that a splice acceptor is placed at the 5′ end of *lacZ*. Integration of the construct into introns of genes in the correct orientation should create a spliced *lacZ* fusion transcript and a functional fusion protein when the reading frame is maintained. Evidence for this came from experiments in which a construct lacking the 5′ *En-2* splice acceptor yielded 12-fold fewer *lacZ*-expressing transformants than the construct carrying the splice acceptor (*8*). Since the frequency of obtaining integration events that lead to β-galactosidase expression was found to be low, we confined our screen to ES cell transformants that expressed *lacZ*. We anticipated that such a selection would target constitutively expressed genes as well as developmentally regulated genes.

The enhancer trap vector was electroporated (*9*) into the male ES cell line D3 (*10*). Sixty independent G418-resistant ($neo^R$) ES cell lines were established and were tested for *lacZ* expression. Six of these lines expressed *lacZ* in ES cells. By DNA blot analysis, ~70% (11 of 16 tested) were found to contain one to two copies of the intact *lacZ* gene, indicating that as many as one in seven $neo^R$ colonies express *lacZ*. This high incidence of *lacZ* expression in stem cells may indicate that we selected in part for integration events into loci that are active in ES cells because expression of *neo* was required to establish the cell lines.

A total of 600 $neo^R$ ES cell colonies were obtained with the gene trap vector. Replica plating experiments (*11, 12*) revealed that ten colonies expressed *lacZ*; eight of these were established as ES cell lines. The subcellular localization of the β-galactosidase activity varied among the different ES cell lines obtained (Fig. 2 and Table 1). This finding strongly supports the idea that *lacZ* is expressed as a fusion protein in which the protein domains of the endogenous gene have directed the *lacZ* fusion product to different compartments of the cell.

To analyze the expression of the *lacZ* reporter constructs during embryogenesis, we generated chimaeric embryos (Table 1). Different chimaeric individuals produced from any particular cell line showed identical patterns of *lacZ* expression although the ratio of staining to nonstaining cells varied with the extent of ES cell contribution (Fig. 2G). This is in accordance with the known extensive cell mixing that occurs during early postimplantation development (*13*). Two enhancer trap lines that expressed *lacZ* in ES cells and five lines that did not were injected into blastocysts and analyzed. All eight of the gene trap lines were introduced into blastocysts, one of which did not produce chimaeras as assessed by eye pigmentation at day 12.5 and therefore was not analyzed further.

A variety of *lacZ* activation patterns were observed in chimaeras generated from the various cell lines. Four of the five enhancer trap cell lines that did not express the *lacZ* gene in ES cells also showed no expression in day 9 embryos and were not examined further. In three gene trap cell lines, *lacZ* expression was detected in all tissues of chimaeric embryos at the stages examined. The remaining seven cell lines exhibited developmentally regulated patterns of *lacZ* expression. One enhancer trap cell line expressed the *lacZ* gene in ES cells but not in day 9 or day 12.5 chimaeric embryos. Three gene trap cell lines showed widespread staining but some tissues were clearly devoid of β-galactosidase activity (Table 1). The remaining three cell lines (GT-10, D3-3-15, and D3-6-28) displayed well-defined spatially restricted patterns of expression (Fig. 2). In the most striking of these, D3-6-28, expression was confined to the posterior neuropore at day 8.5 and then extended further anterior in the spinal cord with progressive development.

To begin to test for germ line transmission of these reporter constructs, D3-6-28 cells were used to produce 17 chimaeric

**Table 1.** Expression of β-galactosidase in chimaeric embryos. Chimaeric embryos were produced by injecting albino CD-1 (Charles River, Quebec) blastocysts. Postimplantation embryos were dissected from the uterus and stained for β-galactosidase activity (*12*). Chimaerism was detected in day 12.5 embryos by scoring the pigmentation of the eyes. The D3-3, D3-6, and GT series of cell lines were obtained with p3LSN, p6LSN, and pGT4.5, respectively (Fig. 1). Visible differences in β-galactosidase (β-gal.) activity (− to +++) are shown. CNS, central nervous system.

| Cell line | *LacZ* expression | | | | |
|---|---|---|---|---|---|
| | In stem cells | | In embryos | | |
| | β-gal. activity | Subcellular localization | Age of embryos (days) | No. staining/ no. analyzed | Pattern of staining |
| D3-3-3 | − | | 9.5 | 0/22 | |
| D3-3-8 | − | | 9.5 | 0/37 | |
| D3-3-15 | − | | 9.5<br>10.5<br>12.5 | 13/33<br>8/11<br>9/11 | Spatially restricted, see Fig. 2 |
| D3-6-1 | − | | 9.5 | 0/19 | |
| D3-6-4 | − | | 9.5 | 0/18 | |
| D3-3-10 | + | Cytoplasm | 9.5<br>12.5 | 0/33<br>0/10 | |
| D3-6-28 | + | Cytoplasm | 5.5<br>7.5<br>9.5<br>10.5<br>12.5 | 2/3<br>0/19<br>15/18<br>10/13<br>10/12 | Spatially restricted, see Fig. 2 |
| GT-1 | +++ | Cytoplasm (single dot) | 9.5<br>12.5 | 7/9<br>13/15 | Constitutive |
| GT-2 | ++ | Nuclear | 9.5<br>12.5 | 6/8<br>9/9 | Constitutive |
| GT-4-2 | + | Nuclear | 12.5 | 9/13 | Constitutive |
| GT-4-1 | + | Cytoplasm (single dot) | 9.5<br>12.5 | 5/9<br>12/15 | Restricted in CNS, absent from heart and liver |
| GT-6 | + | Cytoplasm | 12.5 | 7/11 | Widespread but absent in CNS |
| GT-8-1 | + | Cytoplasm | 9.5<br>12.5 | 0/9<br>13/21 | Widespread but absent in CNS |
| GT-10 | ++ | Cytoplasm (vesicles) | 8.5<br>10.5<br>12.5 | 4/13<br>7/14<br>7/11 | Spatially restricted, see Fig. 2 |

animals (*14*). Of eight fertile male chimaeras test-bred, six transmitted the ES genotype into progeny. Embryos derived from one germ line chimaeric male were tested for expression of the reporter gene and *lacZ* staining was found in a pattern identical to the one observed in chimaeras. This demonstrates that patterns of reporter gene expression in chimaeras can truly reflect expression in transgenic animals.

In a sample of only 14 ES cell lines carrying the *lacZ* reporter constructs, we readily observed developmental regulation of the reporter gene. The fact that six of these lines expressed *lacZ* in ES cells suggests that selection for genes active in ES cells is a valid approach to identify genes expressed in a tissue-specific or spatial pattern at different stages of embryogenesis.

The enhancer trap and gene trap approaches are based on the assumption that the expression pattern of the reporter gene reflects the expression of the endogenous host gene. Whether this assumption is correct will only become apparent after cloning of the host genes at the site of integration. However, the use of ES cells instead of transgenic mice should simplify the cloning of the host genes. For the enhancer trap, the simple structure of the integration site as well as the inclusion of the $SUIII^+$ gene should facilitate rapid cloning of the host flanking sequences. For the gene trap construct, it should be possible to clone the exon sequences of the endogenous gene present in the *lacZ* fusion transcript directly from cDNA and to purify the fusion proteins from cell extracts by means of antibodies directed against β-galactosidase.

Expression patterns provide clues as to the function of developmentally regulated genes but final proof will come from analyzing mutant phenotypes. Since the enhancer trap vector must integrate near cis-acting regulatory sequences, we expect that some integration events will cause a mutation in an endogenous gene. Insertions of the gene trap vector should in all cases produce a mutation in the host gene. The use of ES cells affords a unique opportunity to study both dominant and recessive mutations obtained from such integration events. Dominant embryonic lethal mutations can be studied directly in chimaeric embryos and recessive mutations can be studied after germ line transmission. Thus our results show that introducing *lacZ* reporter constructs into ES cells can provide an efficient tool to search for and mutate mouse genes expressed in developmental patterns and should ultimately lead to the identification of novel genes involved in the regulation of mammalian development.

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18. A.G. and W.C.S. contributed equally to this work. We thank B. A. Auerbach and V. Maltby for technical assistance, A. Bernstein and H. Clarke for helpful discussion, R. Kemler for providing the D3 cell line, and N. G. Miyamoto for the human β-actin promoter fragment. This work was funded by grants from the Medical Research Council (MRC) and National Cancer Institute (NCI) of Canada to A.J. and J.R. A.J. is an MRC Scholar and J.R. is an NCI Research Scientist. A.G. was supported by a fellowship from the Deutsche Forschungsgemeinschaft and W.C.S. by an NCI of Canada studentship.

14 December 1988; accepted 10 February 1989