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Patterns of expression of position-dependent integrated transgenes in mouse embryo

(development/β-galactosidase/cell markers/compartmentalization)

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ABSTRACT **The abilities to introduce foreign DNA into the genome of mice and to visualize gene expression at the single-cell level underlie a method for defining individual elements of a genetic program. We describe the use of an *Escherichia coli lacZ* reporter gene fused to the promoter of the gene for hypoxanthine phosphoribosyl transferase that is expressed in all tissues. Most transgenic mice (six of seven) obtained with this construct express the *lacZ* gene from the hypoxanthine phosphoribosyltransferase promoter. Unexpectedly, however, the expression is temporally and spatially regulated. Each transgenic line is characterized by a specific, highly reproducible pattern of *lacZ* expression. These results show that, for expression, the integrated construct must be complemented by elements of the genome. These elements exert dominant developmental control on the hypoxanthine phosphoribosyltransferase promoter. The expression patterns in some transgenic mice conform to a typological marker and in others to a subtle combination of typology and topography. These observations define discrete heterogeneities of cell types and of certain structures, particularly in the nervous system and in the mesoderm. This system opens opportunities for developmental studies by providing cellular, molecular, and genetic markers of cell types, cell states, and cells from developmental compartments. Finally this method illustrates that genes transduced or transposed to a different position in the genome acquire different spatiotemporal specificities, a result that has implications for evolution.**

To understand how the mammalian body is formed during embryogenesis, a complete evaluation of cell diversity is needed. This requires insight into the concept of cell type and cell position. At the molecular level this diversity is reflected by differences in the genetic program adopted. The programs are determined by a series of developmental decisions taken during embryogenesis as the result of a combination of positional information, cell interactions, and lineages. Cellular and molecular markers are operational in defining components of the genetic program and, therefore, cell identity and cell diversity. Markers have been produced in mammals, but for many of them whether they map to a single gene is unknown.

Two technical advances provide a way of producing additional markers and studying the genetic organization of the genome. (*i*) Genetic material can now be introduced into the genome of mice (1, 2). (*ii*) By using the *Escherichia coli lacZ* gene as a reporter, gene expression can be visualized at the cell level (3, 4) and in whole mounts (5, 6). We show here that, in mice as in *Drosophila* (7–9), certain *lacZ* constructs can serve to detect control elements by complementation in the genome. We use a reporter gene fused to the promoter of a gene expressed in all tissues. This construct generates β-galactosidase (β-Gal) expression patterns that are position dependent. The patterns are found at high frequency (almost 100%) in transgenic mice in contrast to other studies (refs. 5 and 10, but see also ref. 11). If, as in *Drosophila* (12), the β-Gal patterns indicate the activity of the flanking DNA (13), our observations suggest that the genome is mainly organized into functional domains with ontogenic specifications. Therefore, this method generates a large set of specific cell and molecular markers for developmental studies.

MATERIALS AND METHODS

The *HPRT-nls-lacZ* Gene, Transgenic Mice, and Cells. The 1.7-kilobase (kb) *Eco*RI–*Xma* III fragment (for HPRTnlsLacZ-1 to 4 transgenic lines, where nls represents simian virus 40 large tumor nuclear location signal) or the 0.4-kb *Xma* III fragment (for HPRTnlsLacZ-5 to 7 transgenic lines) of the promoter of the hypoxanthine phosphoribosyl transferase (HPRT)-encoding gene (14) was ligated to pM-MuLVnlsLacZΔEnh (4), (in which MuLV represents Moloney murine leukemia virus) linearized with *Sal* I and treated with Klenow enzyme, resulting in pM-MuLVHPRTnlsLacZΔEnh. The enhancers of the long terminal repeat 3′ were deleted from pM-MuLVnlsLacZΔEnh as described (15). The DNA insert was obtained by using the *Pst* I restriction site and was purified on glass beads. Manipulation of mice and eggs and microinjection was done as described (16). The male pronuclei of fertilized eggs from females (C57BL/6 × DBA/2) mated with F_1 males of the same strain were injected with 500 copies of the HPRTnlsLacZ insert (Fig. 1*B*). The founders were mated with (C57BL/6 × DBA/2) F_1 animals. Stocks of transgenic animals were maintained by intercrosses. Cells in culture received 100 copies of the HPRTnlsLacZ insert or of pSVnlsLacZ. Cell lines are referenced elsewhere (3, 17).

β-Gal Expression Analysis. For embryos, the day that the copulation plug was noticed was taken as embryonic day 0 (E0). Postnatal animals are denoted by P. Whole embryos were fixed for 30 min at 4°C in 4% para-formaldehyde/phosphate-buffered saline (PBS), washed twice with PBS, and incubated for 18 hr at 30°C with a reaction mixture containing 4-chloro-5-bromo-3-indolyl β-galactoside (X-Gal) at 0.4 mg/ml (3, 18).

Coronal and sagittal tissue cryostat sections were obtained from pieces of embryos or tissues equilibrated in 30% (wt/vol) sucrose/PBS. Cytofluorometric analysis was performed as in ref. 19.

Northern (RNA Blot) Analysis. Five micrograms of poly-$(A)^+$ RNA isolated from embryos or cells in culture was fractionated by electrophoresis on 1% agarose/formaldehyde

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Abbreviations: HPRT, hypoxanthine phosphoribosyl transferase; β-Gal, β-galactosidase; E and P followed by a number indicate embryonic and postnatal days, respectively; X-Gal, 4-chloro-5-bromo-3-indolyl β-D-galactoside.

gel, transferred to nylon sheets, and hybridized as described (20).

RESULTS AND DISCUSSION

Very High Efficiency of Generation of Different β-Gal Patterns in HPRTnlsLacZ Transgenic Lines. The basic reporter element used in this work was the *E. coli lacZ* gene fused to sequences coding for a nuclear location signal of the simian virus 40 (SV40) large tumor protein and to a polyadenylylation signal from Moloney murine leukemia virus (4). It was combined with the promoter of the gene for HPRT (Fig. 1*B*), which codes for a constitutively expressed enzyme (14). In accordance with this constitutive expression, Northern (RNA) blot analysis of embryonic and adult tissues with a HPRT probe revealed similarly high levels of RNA (Fig. 1*A*).

Three observations suggest that HPRTnlsLacZ DNA insert is appropriate for the generation of markers that define elements of a genetic program. (*i*) The DNA is actively transcribed when introduced by microinjection into different cell types (Table 1) and in oocytes, two-cell and four-cell embryos (data not shown) at a level comparable to that obtained with a construct in which the early promoter of simian virus 40 drives *nls-lacZ*. This result shows that the former construct contains a functional promoter the activity of which is independent of a tissue-specific transactivator. (*ii*) In apparent contradiction with the previous observation, in transgenic lines (Table 2 and Fig. 2) HPRTnlsLacZ insert is expressed in only a few tissues, and there are no similarities between the β-Gal patterns generated (Fig. 2*G*). To determine whether the tissues that are negative for X-Gal histochemical staining express low levels of β-Gal, the more sensitive fluorescein di-β-D-galactopyranoside cytofluorometric analysis was used (19). The cells from X-Gal-negative tissues were indistinguishable from control cells (Fig. 3, main peaks). In contrast, cells from X-Gal-positive territories gave positive signals (Fig. 3 *A* and *C*, peaks with mean fluorescence of 1057 and 2264). Therefore, when integrated into the genome HPRTnlsLacZ insert by itself is insufficient for constitutive expression. (*iii*) The transcripts in X-Gal-positive cells initiate in the HPRT promoter (Fig. 1*A*). These three observations make it likely that the HPRT promoter needs to be complemented by elements of the genome that act at a distance for expression to take place. We hypothesize that the minimal function required for these elements is to make accessible a genetic domain to transcriptional factors. These elements may or may not, in addition, enhance the HPRT promoter. Their main property is that they confer on HPRTnlsLacZ insert a specific spatiotemporal pattern of expression in each transgenic animal.

Such a hypothesis may explain the unusually high efficiency with which β-Gal patterns were obtained (six of seven transgenic lines). The lower efficiency (20%) reported with weak viral promoters in recent studies (5) may be due to an additional requirement for an enhancer. Further molecular analysis should provide information on the properties of the

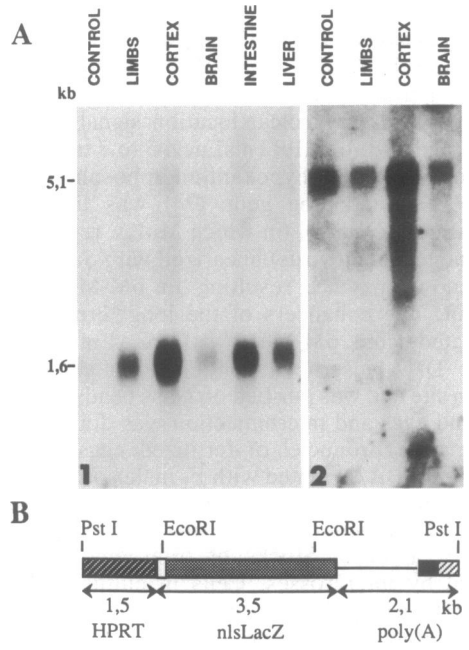

FIG. 1. Expression of HPRT and *HPRT-nls-lacZ* genes in various organs. (*A*) RNA blot analysis of HPRT and HPRTnlsLacZ transcripts. From 0.2 μg to 5 μg of poly(A)$^+$ RNA were fractionated on 1% agarose/formaldehyde gel, transferred to nylon, and hybridized with one ^{32}P-labeled probe of the following: (*1*) HPRT cDNA (1-day exposure) or (*2*) LacZ (5-day exposure). (*1*) Lanes: control, HPRT-nlsLacZ BRL cells (0.2 μg); limbs, forelimbs and hindlimbs (5 μg); cortex, cortex (5 μg); brain, cortex and basal ganglia (1 μg); intestine, digestive tract (5 μg); liver, liver (5 μg). The size of mouse HPRT RNA is 1.6 kb. (*2*) Lanes: control, HPRTnlsLacZ BRL cells (0.2 μg) transcribing *lacZ* from the HPRT promoter; limbs, forelimbs and hindlimbs (5 μg); cortex, cortex (5 μg); brain, cortex and basal ganglia (1 μg). Limbs and cortex tissues are from HPRTnlsLacZ-1 transgenic line; brain, intestine, and liver are from HPRTnlsLacZ-3 transgenic line. The expected size of RNAs initiating in the HPRT promoter is 5.1 kb (see control lane and *B*). (*B*) Diagram of HPRT-nlsLacZ construct. The HPRT promoter (▬) was a 1.5-kb *Pst* I–*Xma* III fragment of the genomic clone. nlsLacZ sequence came from pM-MuLVnlsLacZΔEnh (4). □, nuclear locating signal; ▬, LacZ. The polyadenylylation signal (—▬) was from Moloney murine leukemia virus.

Table 1. Expression of the HPRTnlsLacZ insert in cultured cells

Cell line	Type	β-Gal$^+$ cells,* % HPRT	β-Gal$^+$ cells,* % SV
NS 20 Y	Neuronal	5	28
G26	Glial	12	19
3/A/I-DI	Osteogenic	28	42
PCD1	Myocardial	17	39
1168	Fibroblastic	16	51
PYS-2	Endodermal	5,4	8,9
BRL	Hepatocyte	19	9
Fa0	Hepatocyte	6	6

*One hundred copies of the *Pst* I fragment of HPRTnlsLacZ (HPRT) or of pSVnlsLacZ (SV) were microinjected into the nucleus of various cell types. Results are expressed as percentage of microinjected cells detected as β-Gal$^+$ by X-Gal staining 36 hr after microinjection. Total number of microinjected cells is 400 (two to three independent experiments).

Table 2. HPRTnlsLacZ-1 to -6 transgenic lines

Line	Copy,* no.	Expression† Pre-I	Expression† Post-I	Expression† Adult
1	9	−	+	+
2	12	−	+	+
3	1	−	+	+
4	2	+	ND	ND
5	16	−	+	−
6	2	−	−	−

The *Pst* I DNA fragment of HPRTnlsLacZ was used to prepare transgenic mice. Southern blotting and progeny analysis indicated that *lacZ* had integrated into 7 of the 40 mice born. In all lines except for line 5, the transgene is at a single chromosomal site.
*Number of *lacZ* copies varied from 1 (HPRTnlsLacZ-3) to 16.
†Expression determined by X-Gal staining was obtained in six of seven transgenic mice. Pre-I, preimplanation embryos; Post-I, postimplantation embryos; ND, not determined.

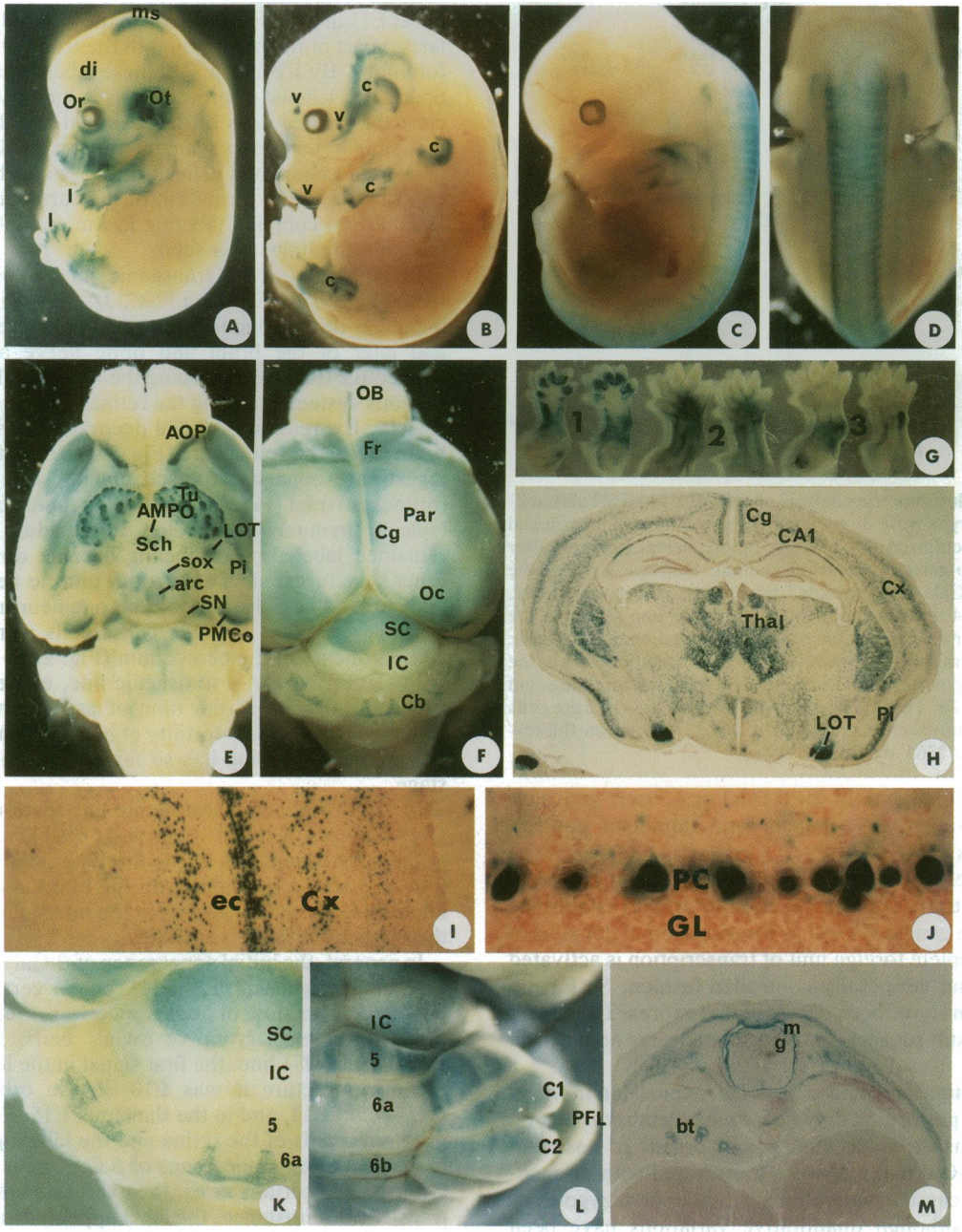

FIG. 2. β-Gal patterns in transgenic embryos. E13–E14 embryos (*A–D*), organs from postnatal mice (*E*, *F*, *G*, *K*, and *L*), and cryostat tissue sections (*H*, *I*, *J*, and *M*) were stained with X-Gal to reveal β-Gal activity. At least 15 β-Gal$^+$ animals from each line were examined. (*A*) HPRTnlsLacZ-1 transgenic line (E14) expressed the transgene in the head [orbita (Or), otic structures (ot), mouth, mesencephalon (ms), and diencephalon (di)], in the forelimbs, hindlimbs, and interdigits (l), and in the spinal cord and ganglia. (*B*) HPRTnlsLacZ-2 transgenic line (E14) expressed the transgene in the head [cartilage (c) of cranial structures, mesenchyme surrounding the vibrissae (v) and cartilage (c) of the skeleton, including forelimbs and hindlimbs]. (*C*) HPRTnlsLacZ-3 transgenic line (E13) expressed the transgene in the meninges of the spinal cord and in the tail and envelope of the hindlimbs and forelimbs. (*D*) Dorsal view of HPRTnlsLacZ-3 mouse showing the segmented pattern of the meninges of the spinal cord at E13. (*E*, *F*, and *H–K*) HPRTnlsLacZ-1 brain (P11). (*E*) Ventral view of the brain. The structures expressing the transgene are the anterior olfactory nucleus (AOP), olfactory tubercule (Tu), nucleus of the lateral olfactory tract (LOT), piriform cortex (Pi), substantia nigra (SN), suprachiasmatic nucleus (SCh), anterior medial preotic nucleus (AMPO), supraoptic decussation (sox), arcuate hypothalamus nucleus (arc), and posteromedial cortical amygdaloid nucleus (PMCo). (*F*) Dorsal view of the brain. The transgene is expressed in the frontal (Fr), occipital (Oc), cingulate (Cg), and temporal cortex, in the superior colliculus (SC), and in some cerebellar lobules (Cb). (*G*) *In toto* staining of a forelimb and a hindlimb of HPRTnlsLacZ-1, -2, and -3 at E15. (*H*) Coronal section through the hippocampus and the lateral olfactory tract. The transgene is expressed in certain layers of the cingulate (Cg), retrosplenial, frontal, parietal and piriform (Pi) cortex; in the caudate putamen (CPu), in numerous nuclei of the thalamus (Thal) and of the hypothalamus and in field CA1 of Ammon's horn. (*I*) Coronal section through the cortex (Cx). Three laminar structures express the transgene; the external capsule (ec) is indicated. (*J*) Section through the cerebellum at the level of a compartment of Purkinje cells (PC) expressing the transgene. GL, granular layer. (*K*) Dorsal view of the cerebellum of HPRTnlsLacZ-1 transgenic line. Areas expressing the transgene are in the vermis and in lobules (5, 6a, and 6b). (*L*) Dorsal view of the cerebellum of HPRTnlsLacZ-2 transgenic line. Areas expressing the transgene are organized in compartments in all lobules (5, 6a, and 6b) and in crus 1 (C1) crus 2 (C2), and paraflocculus (PFL). (*M*) Transverse section through the spinal cord, lungs, and liver of HPRTnlsLacZ-3 transgenic line at E14. The arachnoid mater and pia mater (m), glial cells (g), and cells of the smooth muscles in the bronchial tractus (bt) are β-Gal$^+$.

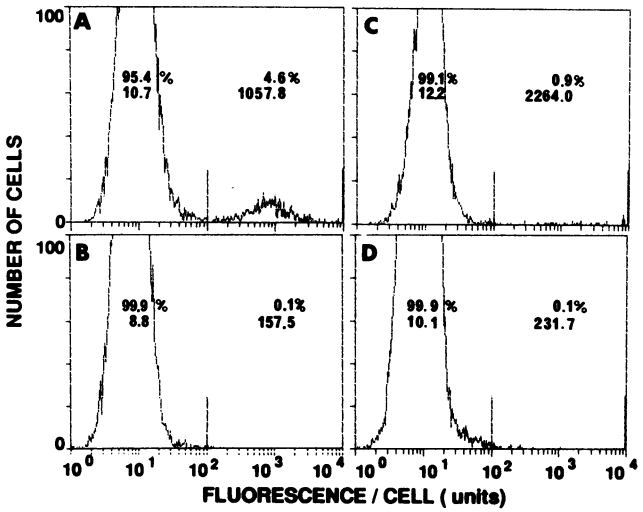

FIG. 3. Cytofluorometric analysis of cells of E14 HPRTnlsLacZ-1 embryos. Embryos were dissociated with a solution of trypsin and collagenase and cultured for 2 weeks before being analyzed after fluorescein di-β-D-galactopyranoside staining. During this period the percentage of β-Gal$^+$ cells revealed by X-Gal staining was stable. β-Gal$^+$ embryo (*A* and *C*) and β-Gal$^-$ embryo (*B* and *D*) are of the same litter. Upper numbers in the peaks indicate percentage of cells, and lower numbers indicate mean fluorescence value of cells in the peak. Cells are from the snout (*A* and *B*) and from the forelimbs and hindlimbs (*C* and *D*). The main peaks with mean of 8.8–12.2 are cells exhibiting autofluorescence. Peaks in *A* and *C* with mean fluorescence of 1054 and 2264 are β-Gal$^+$ cells.

complementing elements of HPRTnlsLacZ insert and their relation to far-upstream-enhancer and dominant control-region elements (21–23).

Whatever the precise molecular mechanism, the generation of β-Gal patterns (refs. 5, 10, and this article) establishes that the genome can be viewed as a succession of domains in which an incomplete foreign unit of transcription is activated in a spatially and temporally controlled fashion. So far, no transgenic strains have been obtained that express LacZ in all tissues. This result suggests that few domains are active in all tissues.

The β-Gal Patterns. Analysis of β-Gal expression in transgenic mice was performed on whole-stained embryos and on cryostat sections of whole embryos or organs of postnatal animals. The β-Gal patterns were transmitted with no modifications to successive generations, whether hemizygotes or homozygotes, although quantitative variations have been noticed (data not shown). The transgene corresponded to a single genetic locus as determined by crosses in all lines, except HPRTnlsLacZ-5 transgenic line.

Of the seven transgenic founders one, which expressed LacZ in mesenchymal tissues, died before breeding. In HPRTnlsLacZ-6 transgenic line, no expression of LacZ was ever detected. It is not known whether the structure of the two *lacZ* copies is intact. We report on the five other transgenic lines.

In HPRTnlsLacZ-1 line, expression was detected in embryos at E9, mainly in mesodermal derivatives. The labeling pattern at E14 (Fig. 2*A*) included the pinna, otic structures, structures in the face, the orbita, the limbs, and the fingers. In addition, neurons of the nervous system (in the diencephalon, basal ganglia and spinal cord, and spinal and cranial ganglia) expressed LacZ. In the adult the β-Gal neuronal pattern in the central nervous system became complex (Fig. 2 *E*, *F*, *H*, *I*, and *K*). β-Gal$^+$ cells belonged only to neuronal cells, but they were of various types—stellate and pyramidal (Fig. 2*I*), Purkinje (Fig. 2*J*), amacrine, and ganglionic cells.

In HPRTnlsLacZ-2 transgenic line, expression was apparent at E11 in the telencephalon in the choroid plexuses, on the lateral part of the mouth, and in the trunk at the bases of the forelimbs. By E14, (Fig. 2*B*) these labeled groups had developed. The labeled choroid plexuses had invaded the ventricles; the labeled cells of the face had invaded sagittally the mandibular territories, surrounding the vibrissae, and the labeled mesenchymal cells at the bases of the forelimbs had contributed to the formation of the cartilage of the scapula. In addition, complex labeling in the cranial skeleton of the face and the limbs was seen.

In HPRTnlsLacZ-3 transgenic line, expression was detected at E9. At E13 (Fig. 2 *C*, *D*, and *M*), it included the meninges of the spinal cord (arachnoid mater and pia mater) and later of the brain and optic nerve and cells of the connective tissues enveloping the whole body. In the central nervous system including the retina, a class of glial cells was β-Gal$^+$. Expression of β-Gal decorated all structures of the brain and of the spinal cord (Fig. 2*M*). Smooth muscles of the digestive (esophagus, stomach, and intestine) and respiratory (bronchus) tract (Fig. 2*M*) and of the tunica of various organs (spleen, thymus) were labeled. In the adult, these cell types remained labeled, and labeling became obvious in certain other structures, such as smooth muscle cells in the ureter and in blood vessels. In addition, one neuronal cell type, the Purkinje cell, was β-Gal$^+$. These cells were distributed in compartments in the cerebellum (Fig. 2*L*).

In HPRTnlsLacZ-4 transgenic line, expression started in two-cell embryos at the time of activation of the zygotic genome. The very low fertility of both the female founder and of one male offspring has so far limited our studies to this stage.

In HPRTnlsLacZ-5 transgenic line (data not shown), discrete patterns of β-Gal$^+$ cells were visible at E14 in the ear, at the inferior junction of the forelimb, and in the interdigits (but only of the hindlimbs). In the brain, a coherent sagittal line of β-Gal$^+$ cells was seen in the internal germinal layer of the epithelium in the ventricle.

In general, the initial appearance of β-Gal$^+$ cells was during early embryogenesis (E8–E11), but in certain cases it was postnatal. This time of appearance was a reproducible characteristic of each group of β-Gal$^+$ cells. For instance, in HPRTnlsLacZ-1 line, the first signal in the legs was at E9, in the otic structure it was E10, in the orbita E12, in the cerebellum E14, and in the thalamus E15. For amacrine and ganglionic cells of the retina and the labeling of the neurons in the neocortex, appearance of β-Gal$^+$ cells was a late event (after P3). In certain cases, the expression of LacZ was only transient. For instance, the label appeared early in the motor neurons of the spinal cord in HPRTnlsLacZ-1 transgenic line (E14) and disappeared rapidly after birth. In contrast, other postmitotic multipolar neurons of the same transgenic line stably expressed LacZ. For example, in the midbrain and forebrain (in the cortical layers) β-Gal$^+$ cells were still detected in 1-year-old animals in a pattern identical to the one illustrated in Fig. 2 *E–K*.

It is interesting to correlate the patterns of expression to the basic parameters of development. There are three ways of describing development: (*i*) by cell type (related to function); (*ii*) by topography (related to position); and (*iii*) by lineages (related to origin).

Certain β-Gal patterns can be interpreted as reflecting the expression of a conventional cell-type marker. For instance, in HPRTnlsLacZ-3 transgenic line all smooth muscle cells are β-Gal$^+$ irrespective of their location. Similarly, the meninges and a class of glia are uniformally labeled with no apparent topographic restriction. For these cells the *lacZ* gene becomes a specific marker that can be used to follow and to isolate them as pure population (6, 19) from the time they begin to express LacZ. A striking observation is that almost

all the β-Gal patterns in any one transgenic line are constituted by cell types derived from distinct lineages. This cannot be due to multiple sites of insertion of *lacZ* because for all lines the transgene is transmitted as a single Mendelian factor and corresponds (HPRTnlsLacZ-1 and -3 transgenic lines) to a single position of insertion as judged by restriction enzyme analysis. This result may indicate that a given ontogenic domain is active in different tissues and at different times during development. This complex situation may be the rule rather than the exception, as suggested by the pleiotropy of a number of mutants and by the complexity of the patterns of expression of certain markers. Also noticeable is the frequent presence in the patterns of elements of the nervous system (four of six) and mesodermal derivatives (four of six, Table 2); this probably reflects the complexity of gene expression required for the development of these organs. The molecular analysis of the relevant genetic domain will indicate whether this property is due to the presence of a gene with pleiotropic functions or due to two or more genes in the same domain or caused by the fusion of two domains as a result of rearrangements during integration.

Other β-Gal patterns can be tentatively interpreted as corresponding to topographical labeling because they define compartments within a given tissue that would not have been predicted from traditional embryological description. Examples include a small group of cells at the base of the forelimbs in HPRTnlsLacZ-5 and the Purkinje cells in HPRTnlsLacZ-3 line (Fig. 2*L*). Also, in HPRTnlsLacZ-1 transgenic line the *lacZ* gene is expressed in neuronal cells in only certain structures (Fig. 2 *H* and *K*) and in mesenchymal cells in only certain organs (Fig. 2*A*). One characteristic of this topographical label is that it is also restricted to certain cell types. This double restriction (certain localizations of certain cell types) generates a fine tissue heterogeneity. One example is the pyramidal cells in Ammon's horn (Fig. 2*G*) and the Purkinje cells in the cerebellum (Fig. 2 *K* and *L*). The expression of LacZ in these cells defines parasagittal compartments in certain lobules but with a different geometry in different lobules. Parasagittal compartmentalization detected by immunological and biochemical markers (24) has been reported in the cerebellum, but these patterns are different from the β-Gal patterns of HPRTnlsLacZ-1 and -3 lines. Finally, we did not find patterns that strictly corresponded to lineage labeling.

The large number of different β-Gal patterns generated suggests little restriction of expression by a particular tissue. In addition, the marker is not restricted to actively dividing cells (because postmitotic neurons are labeled). Further analysis of patterns of expression will open opportunities for developmental studies. (*i*) This analysis will assist in defining more completely the repertory of heterogeneity, a prerequisite for the study of brain and mesoderm development. (*ii*) Further work will help the analysis of the organization of tissues and organs as defined by developmental compartmentalization. (*iii*) By tracing the origin of the β-Gal$^+$ cells during embryogenesis, such analysis will provide information on the mechanism by which these compartments are generated. (*iv*) In combination with transgenic animals obtained with promoters exhibiting cell-type specificities (18, 25) this approach will provide markers for all important cell types, cell states, and cells in a given developmental compartment. (*v*) Finally, these results illustrate how the expression of a gene transduced or transposed in an ontogenic domain acquires different spatiotemporal properties. This capability is clearly instrumental in increasing the plasticity of the genetic material, an observation that has implications for evolution.

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