Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 7357–7361, August 1995
Medical Sciences

WW6: An embryonic stem cell line with an inert genetic marker that can be traced in chimeras

(DNA *in situ* hybridization)

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Communicated by Salome G. Waelsch, Albert Einstein College of Medicine, Bronx, NY, May 8, 1995

ABSTRACT **Mutant mice produced by gene targeting in embryonic stem (ES) cells often have a complex or embryonic lethal phenotype. In these cases, it would be helpful to identify tissues and cell types first affected in mutant embryos by following the contribution to chimeras of ES cells homozygous for the mutant allele. Although a number of strategies for following ES cell development *in vivo* have been reported, each has limitations that preclude its general application. In this paper, we describe ES cell lines that can be tracked to every nucleated cell type in chimeras at all developmental stages. These lines were derived from blastocysts of mice that carry an 11-Mb β-globin transgene on chromosome 3. The transgene is readily detected by DNA *in situ* hybridization, providing an inert, nuclear-localized marker whose presence is not affected by transcriptional or translational controls. The "WW" series of ES lines possess the essential features of previously described ES lines, including giving rise to a preponderance of male chimeras, all of which have to date exhibited germ-line transmission. In addition, clones selected for single or double targeting events form strong chimeras, demonstrating the feasibility of using WW6 cells to identify phenotypes associated with the creation of a null mutant.**

Embryonic stem (ES) cells are widely used for the production of mice bearing a germ-line mutation (1). The phenotypes generated are often difficult to interpret in cellular and molecular terms, especially when the gene inactivated is essential at an early developmental stage. Methods for bypassing lethality in order to identify functions for embryonic lethal genes in adult tissues have been developed (2–4). However, these strategies are tissue specific and hypothesis driven. They may miss unsuspected gene functions. An alternative approach, aimed at identifying tissues affected by a particular mutation, is to determine the contribution of homozygous mutant ES cells to the tissues of a chimera. Markers that have been used to identify the progeny of injected ES cells exploit a glycerophosphate isomerase (GPI) isozyme difference (5, 6), a difference in H2 class 1 molecules expressed at the cell surface (7), or the expression of a constitutively expressed, *lacZ* reporter gene uniquely present in the injected ES cell genome (8). However, each of these approaches has a serious drawback: only tissue extracts, not cell types, can be compared for GPI isozymes; H2 molecules are only weakly expressed in prenatal tissues; and expression of a reporter gene may depend on transcriptional and translational controls. To overcome these problems, we sought an inert genetic marker that could readily be detected in ES cells and in essentially all cell types of the mouse, independent of spatial or temporal constraints on transcription or translation.

The transgenic mouse strain 83 provides an appropriate marker (9). This strain [now termed TgN(Hbb-b1)83Clo; ref. 10] was created by injection of cloned DNA containing the mouse β^{maj} globin gene (11). In the original founder mouse, the transgene integrated as a large, tandem repeat of ≈1000 copies confined to the telomeric region of chromosome 3 (9, 12–15). The transgene is not expressed and no phenotypic effects have been attributed to this insertion (16). Sensitive methods developed to detect the transgene by DNA·DNA *in situ* hybridization using a biotinylated probe have shown the hybridization signal to be precisely localized over cell nuclei (9). This transgene has been used in cell lineage studies of the original transgenic strain (12) and in studies of aggregation chimeras (17, 18). In another experiment, tetraploid embryos derived from strain TgN(Hbb-b1)83Clo were aggregated with 129/Sv blastocyst-derived inner cell mass cells to form chimeras (19).

In this paper, we describe the properties of two ES lines, WW6 and WW1, that were isolated from blastocysts derived from mice carrying the β-globin transgene. *In situ* hybridization experiments show that singly and doubly targeted descendants of WW6 cells can be traced to essentially every nucleated cell type in chimeric embryos. The WW6 line and another ES line carrying the globin transgene have been briefly described (15, 20).

MATERIALS AND METHODS

Isolation of ES Lines. Strain TgN(Hbb-b1)83Clo mice with a single copy of the β-globin transgene (9) were kindly provided by Davor Solter (18). These mice have a mixed genetic background (≈80% C57BL/6J, ≈20% SJL). They were backcrossed twice with 129/Sv^{cc} mice and subsequently intercrossed in order to obtain a strain that carries the β-globin transgene and the rare cc isoform of GPI (GPI^{cc}) at homozygosity. The background of this strain is ≈75% 129/Sv, ≈20% C57BL/6J, and ≈5% SJL.

Matings were initiated on day 0, ovarectomies were performed on day 2, and delayed blastocysts were recovered between days 5 and 10. Several cell lines were derived as described (21) but only WW1 and WW6 cells were characterized for germ-line transmission. WW6 cells were further studied in culture, in tumors, and in chimeras. WW6 cells were shown to be free of mycoplasma by the Genprobe kit (Genprobe, San Diego) and to have 40 chromosomes by karyotype analysis.

Cell Culture. ES cells were cultured on γ-irradiated feeder layers of STO SNL2 cells in α medium (GIBCO) containing 10% ES qualified fetal bovine serum (FBS; GIBCO) and 20 μg of gentamicin per ml (GIBCO) at 37°C in a 5% CO_2 incubator. This STO cell line carries a transfected gene encoding leuke-

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Abbreviations: ES, embryonic stem; LIF, leukemia inhibitory factor; dpc, day(s) postcoitum; GPI, glycerophosphate isomerase.

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mia inhibitory factor (LIF). Mutant WW6 cells that carry one or two copies of a disrupted glycosyltransferase gene included WW6.15, WW6.220, WW6.10, WW6.20, WW6.162, and WW6.186. They were cultured in the same manner as WW6 cells and showed no differences in growth properties under normal culture conditions.

Chimera Production. Blastocysts were obtained from C57BL/6J or MF1 females at 3.5 days postcoitum (dpc) as described (22). WW6 cells were trypsinized, washed once in sterile phosphate-buffered saline at pH 7.2 (PBS), and microinjected into blastocysts. Chimeric progeny were identified on the basis of their brown coat color, and chimeric embryos were identified by the presence of the β-globin transgene in yolk sac DNA.

***In Vitro* and *in Vivo* Differentiation of WW6 Cells.** WW6 cells were harvested with trypsin/EDTA (21) and 2–5 × 10^4 cells were plated onto gelatinized 10-cm plates. After growth for 9 days in α medium containing 20% FBS, 20 μg of gentamicin per ml (GIBCO), and 1000 units of LIF per ml without refeeding, cells were removed in clumps with Dispase (Boehringer Mannheim). Clumps were grown in suspension in α medium containing 20% FBS, 150 μM monothioglycerol (Sigma), and 20 μg of gentamicin per ml (23) and 0.02 μM all-*trans*-retinoic acid (Sigma) to induce differentiation of neurons (24). Culture medium was refreshed every 2 days by replacing one-half of the medium. Embryoid cystic bodies were transferred at day 10 onto gelatinized plates and cultured attached in the same medium. Cultures were visualized with an inverted IMT-2 (Olympus) microscope and photographed.

Tumors were induced in 5-week-old males of the nude strain Crl:cd-1-nuBR (Charles River Breeding Laboratories). Mice were injected subcutaneously with 5 × 10^6 to 10^7 trypsinized WW6 cells that were washed once in sterile PBS. Tumors were fixed in Trump–McDowell fixative consisting of 4% formaldehyde (J.T. Baker), 1% glutaraldehyde, 0.1 M sodium phosphate monobasic, and 0.0675 M sodium hydroxide (pH 7.2) after 3–5 weeks. Sections of 7 μm were stained with hematoxylin/eosin and examined by microscopy.

Genomic DNA Isolation and Southern Blot Analysis. ES cells or yolk sacs were washed with PBS and incubated in 0.7 ml of 50 mM Tris (pH 8.0), 100 mM EDTA, 0.5% SDS, and 0.5 mg of proteinase K per ml (Sigma) at 55°C overnight on a rotator. DNA was extracted once with 0.7 ml of phenol/chloroform (1:1) and chloroform/isoamyl alcohol (24:1), precipitated, and dissolved in 200 μl of 10 mM Tris/1 mM EDTA. Southern blot analysis was performed as described (20). The *Mgat1* gene-specific probe was an ≈0.4-kb exonuclease III fragment from the 3′ end of the *Mgat1* cDNA (20). The β-globin-specific probe was a 650-bp *Hin*fI/*Bam*HI fragment from a β^{dmaj} globin cDNA clone (25) kindly provided by F. Costantini.

***In Situ* DNA·DNA Hybridization.** Chimeric embryos at 14.5 or 16.5 dpc were fixed immediately after dissection in 100% ethanol and 12 M acetic acid (3:1) for >16 hr at 4°C. After two washes in 100% ethanol for 30 min and two washes in xylene for 30 min, they were impregnated with a 1:1 xylene/paraffin mixture overnight at room temperature. Sections of 5–7 μm were mounted on poly(L-lysine)-coated slides, deparaffinized in xylene for 10 min, and rehydrated through serial ethanol dilutions (100%, 90%, 70%, 50%) for 2 min each. After immersion in 2× saline sodium citrate (SSC) for 2 min, slides were treated with RNase A (200 μg/ml; Boehringer Mannheim) for 30 min at 37°C, rinsed with water, and dehydrated through a graded series of ethanol (50%, 70%, 90%, 100%). DNA denaturation and prehybridization were carried out as described (9). The plasmid pMBd2, kindly provided by C. Lo (9), consists of a 7-kb *Eco*RI mouse genomic DNA fragment containing the β-major globin gene (11) with an internal deletion of a 2.5-kb *Bgl* II fragment that contains mouse repetitive DNA elements, subcloned into the pBR322 vector. The pMBd2 plasmid was labeled using the random priming method with biotinylated dUPT (16-mer; Enzo Diagnostics) according to the manufacturer's instructions and used at 0.1 μg/ml final concentration. Hybridization was performed overnight at 42°C followed by two washes with agitation at 50°C in 30% formamide containing 2× SSC for 15 min each and then two washes at 50°C in 2× SSC for 10 min each. Signal was detected using Detek Alk complex (1:200; Enzo Diagnostics) according to the manufacturer's instructions. Sections were briefly counterstained with hematoxylin/eosin Y and mounted with Permount (Sigma).

RESULTS

WW6 and WW1 ES Cells Exhibit a High Degree of Germ-Line Transmission. Newly derived WW1 and WW6 ES lines were found to form strong chimeras when injected into MF1 blastocysts. Ten chimeras from WW1 cells and four from WW6 were 75–100% chimeric based on their content of GPI^{cc} isozyme (data not shown). Two male chimeras derived from WW1 and two from WW6 gave 100% germ-line transmission (9/9, 10/10, 33/33, and 17/17, respectively). Similar results were obtained with later passage WW6 cells and mutants derived from WW6 (Table 1). Each of the clonally derived WW6 cell lines gave about 50% chimeric progeny and *all* chimeric males tested gave germ-line transmission. Therefore, even after numerous passages in culture, selection, and expansion of mutant clones, WW6-derived cells maintained full developmental potential.

WW6 Cells Differentiate into Many Different Cell Types *in Vitro* and *in Vivo*. It is often important to be able to investigate the phenotype of a mutant ES line in a more simple model than the developing mouse. Therefore we investigated the potential of WW6 cells to differentiate into simple and cystic embryoid bodies and to form differentiated cell types in long-term culture of cystic embryoid bodies plated in the absence of feeder cells. Fig. 1 shows that WW6 cells form well-developed cystic embroid bodies with clearly visible blood islands, endothelial vessels, and an outer endodermal cell layer. A large proportion of cystic embryoid bodies exhibited rhythmic beating after day 10 and, by day 15 in retinoic acid, had differentiated into several distinct cells types, including neuronal cells, endothelial tubes, and endodermal cells (Fig. 1). In addition,

Table 1. Chimeras from WW6 cells

Clone	Passage no.	Blastocysts injected	Animals born	Chimeras			♂ tested for germ-line transmission	Germ-line transmission,*	
				Total (%)	♂	♀		>40%, <95%	>95%
WW6	10	51	21	11 (52)	7	4	3	0	3
WW6.15	16	76	41	12 (28)	5	7	4	0	4
WW6.220	16	67	30	13 (43)	10	3	7	1	6
WW6.162	14	29	9	6 (67)	4	2	2	1	1
WW6.186	14	28	11	6 (55)	5	1	3	1	2

*Germ-line transmission was determined on the basis of coat color.

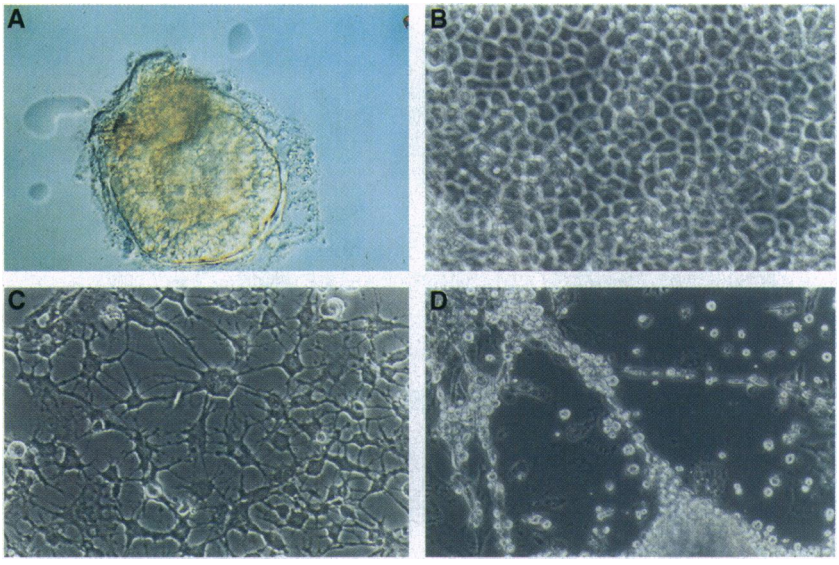

FIG. 1. Morphology of WW6 ES cells differentiated *in vitro*. Differentiation was carried out as described in the text and cells were photographed under phase-contrast. (*A*) Cystic embryoid body after 15 days. Note the clusters of red hematopoietic cells, endothelial blood channels on the surface, and an outer endodermal cell layer. (*B*) Endoderm. (*C*) Neurons. (*D*) Blood channel (endothelium). (*A*, ×63; *B* and *C*, ×630; *D*, ×316.)

muscle cells, mesenchymal cells, and small round cells were observed.

In sections from tumors of WW6 cells produced in nude mice, there were additional tissues and cell types, including focal dyplastic bone with marrow elements, focal osteoid and malignant osteoblasts, neuroectodermal tissue with suggestion of focal neuronal differentiation, mucin-secreting glands with squamous metaplasia and keratin production; malignant cartilage (chondrosarcoma); focally ciliated glandular epithelium; neuroectodermal tissue with true rosettes and pseudorosettes with focal neurons; and adenocarcinoma suggestive of endometrial or gastrointestinal origin.

WW6 Cells Detected at the Single Cell Level in All Tissues of Chimeras. A major reason for generating the "WW" series of ES lines was to be able to follow their contribution to chimeras via detection of the β-globin transgene. Southern blot analyses of genomic DNA extracted from yolk sacs of 16.5-dpc chimeras showed that the transgene can rapidly be detected with a specific globin probe (Fig. 2). It was also detected after more lengthy exposure by a specific, gel-purified probe to the *Mgat1* gene (Fig. 2). This is presumably because of the presence of plasmid sequences in the ≈1000 copies of the transgene. Fortunately, the transgene generates a single hybridizing fragment after digestion with several of the commonly used restriction enzymes (7.0 kb with *Eco*RI, 5.7 kb with *Bam*HI, 4.9 kb with *Hin*dIII, 8.8 kb with *Bgl* II, and 8.6 kb with *Hpa* I; refs. 11, 12, and 20 and data not shown). It can therefore generally be separated from fragments diagnostic for endogenous genes and genes altered by homologous recombination, as shown in Fig. 2 for the *Mgat1* endogenous and disrupted alleles.

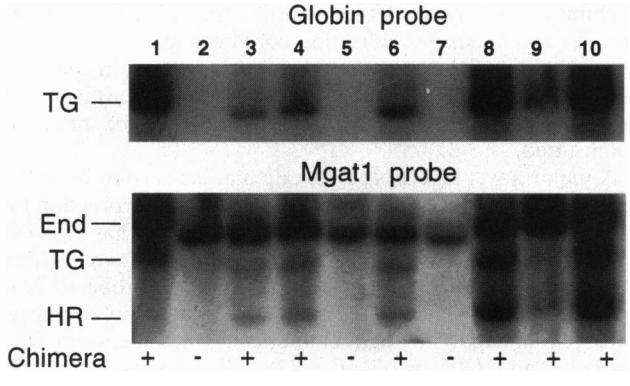

FIG. 2. Detection of chimeric embryos by Southern blot analysis of yolk sac DNA. Host blastocysts were injected with WW6 (lane 1), WW6.15 (lanes 2–7), and WW6.20 (lanes 8–10) ES cells, and embryos were allowed to develop to 16.5 dpc. A Southern blot of yolk sac DNA digested with *Bam*HI was hybridized to the β-globin gene probe (*Upper*) or the *Mgat1* gene probe (*Lower*). Positions of the transgene (TG), endogenous *Mgat1* gene (End), and recombinant *Mgat1* gene (HR) fragments are indicated. The upper blot was exposed for 1 hr with an intensifying screen and the lower blot was exposed for 10 days without an intensifying screen.

The transgene in cells from strain TgN(Hbb-b1)83Clo is readily detected by DNA·DNA *in situ* hybridization (9, 12–14, 16–18). Sections from 9.5-dpc embryos, in which every cell should theoretically carry the transgene, revealed signal in nearly all cells, as expected (Fig. 3 *H* and *I*). At higher magnification it is clear that the transgene was detected as a signal localized to the nucleus and that it was present in only one copy per cell (Fig. 3*G*). This heterozygosity was unexpected because of the attempt to obtain homozygosity of the transgene, but it is consistent with the fact that the transgene segregates in a 50:50 ratio in intercrosses of chimeras formed with WW6 cells (20).

The contribution of WW6 cells to chimeric embryos is shown in Fig. 3 *A–G*. Examination of 7-μm serial sagittal sections from three 14.5-dpc and five 16.5-dpc embryos revealed contribution of WW6 cells to essentially all nucleated cell types. The level of the contribution varied somewhat in different tissues (compare Fig. 3 *A, D,* and *G*). WW6 contributed strongly to brain and small intestine but significantly less to some other tissues in the same embryo. In general, ≥50% contribution of WW6 cells was observed in the cerebral hemisphere, neopallial cortex, ventricular zone, midbrain, spinal cord, hind limb, cardiac ventricular wall, aorta inferior vena cava, atrial wall, thymus, lung parenchyma and bronchi, thyroid, rectum, and small intestine; a contribution of 10–25% was observed in liver parenchyma, choroid plexus and intermediate zone of brain, intercostal muscle, kidney cortex and medulla, adrenal cortex, mandibullar gland, penis, bladder, tail, duodenum, cartilage, and tongue; and a contribution of 1–10% was observed in skin cells, pancreas, adrenal medulla, and nasal and tongue epithelium. These findings probably reflect some degree of incompatibility between WW6 cells and C57BL/6J blastocysts in terms of cell mixing. Strain incom-

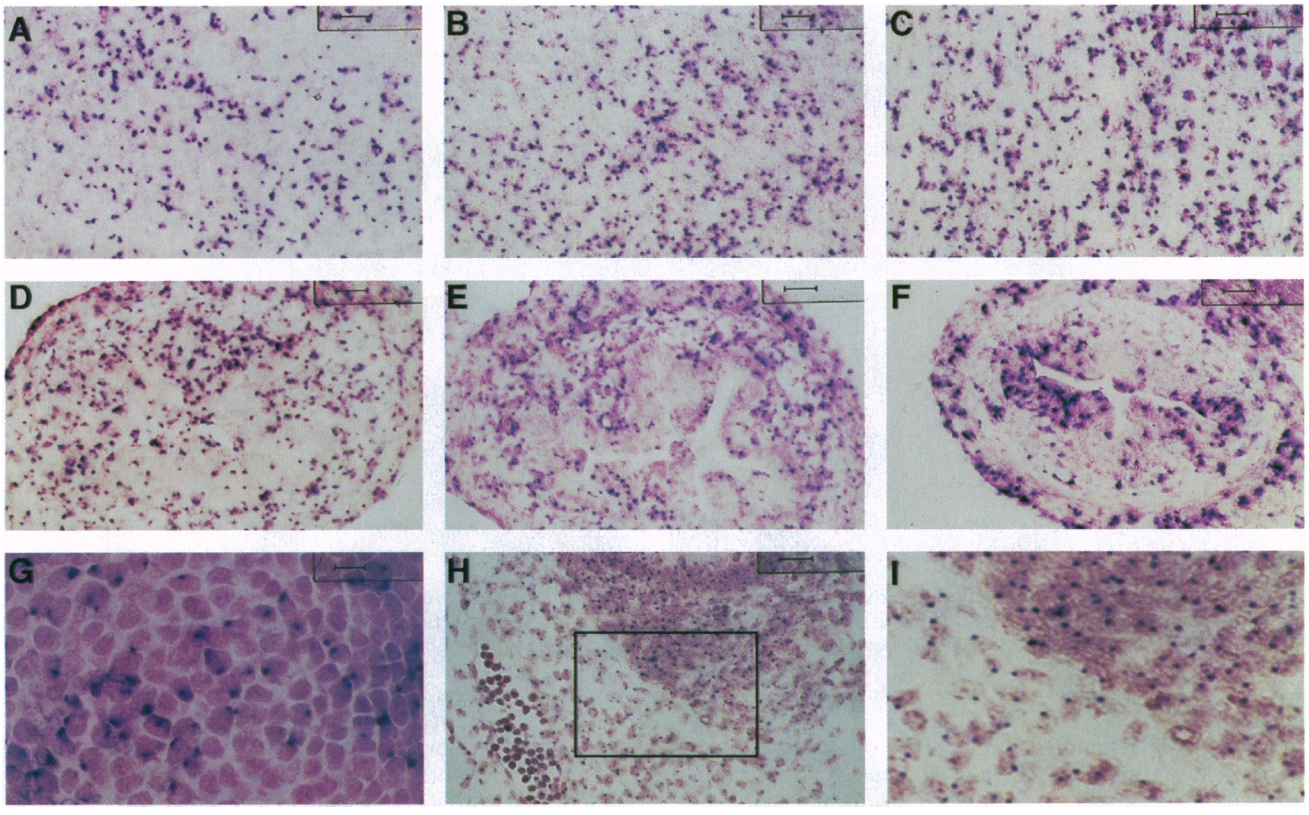

FIG. 3. *In situ* DNA·DNA hybridization of chimeric embryos. WW6 (untargeted, *A*, *D*, and *G*), WW6.15 (singly targeted, *B* and *E*), and WW6.20 (doubly targeted, *C* and *F*) ES cells were injected into C57BL/6J blastocysts, and embryos were analyzed at 16.5 dpc. Sections of 7 μm were hybridized to a biotinylated β-globin gene probe as described in the text and counterstained with hematoxylin/eosin Y. (*A–C*) Midbrain. (Bar = 25 μm.) (*D–F*) Small intestine. (Bar = 25 μm.) (*G*) Cartilage. (Bar = 10 μm.) (*H* and *I*) Five-micron sections through a 9.5-dpc embryo in which all cells carry the transgene. (Bar = 25 μm in *H*; *I* is expansion of the area boxed in *H*.)

patibility of this type has previously been reported to give uneven contribution of cells from particular strain combinations in aggregation chimeras (reviewed in ref. 26). Nevertheless, the data in Fig. 3 show that serial section analysis should readily identify a difference in tissue contribution in chimeras generated from mutant WW6 cells.

If meaningful data are to be obtained from chimeras formed with mutant WW6 cells that have undergone gene disruption(s), cells selected for single and double targeting events must still be able to form strong chimeras. The data in Fig. 3 show that WW6 cells selected through one (Fig. 3 *B* and *E*) or two (Fig. 3 *C* and *F*) rounds of gene targeting are indeed capable of forming chimeras equivalent in strength to unselected WW6 cells (Fig. 3 *A* and *D*). Variation in the degree of contribution of these mutant cells to different tissues was also similar to that observed with WW6 cells. The combined data show that mutant and wild-type WW6 cells can be readily traced in chimeras to essentially all nucleated cell types.

DISCUSSION

The availability of the WW series of ES cell lines provides an approach to identifying specific cell types that are affected by a mutation generated by homologous recombination. Although the genetic background is only 75% 129Sv, genes from 129Sv DNA should be targeted efficiently (27). In fact, the targeting efficiency obtained for seven different genes in three laboratories at the Albert Einstein College of Medicine has varied from 1% to 5% and, in all cases, resulted in high-frequency germ-line transmission (ref. 20; M.B., R. DePinho, and M. Charron, unpublished observations). The generation of homozygous WW6 mutants was readily accomplished from one heterozygous WW6 line (20) but has not been easily achieved at some other targeted loci (R. DePinho and M. Charron, personal communication).

WW6 cells and several clonal derivatives have been shown in this paper to form strong chimeras following their injection into blastocysts. Most importantly, all male chimeras tested gave germ-line transmission and many chimeras transmitted to 100% of their progeny. WW6 cells are easy to maintain in the undifferentiated state and they differentiate into a complex range of cell types that can be studied in tissue culture. Finally, WW6 cells can be tracked to apparently all nucleated cell types in chimeras. The ready detection of the transgene by Southern analysis and *in situ* hybridization could easily be extended to detection by PCR or solution hybridization. Thus, in chimeras as well as other types of *in vivo* assay (e.g., metastasis), the location and number of WW ES cells could be precisely quantitated.

A major advantage of the genetic marker carried by WW6 cells is that it is inert and therefore requires no expression for its detection. Another important advantage is that it is detected *in situ* at the single cell level. Both of these features have also been demonstrated for a satellite DNA probe to *Mus musculus* DNA used to determine the clonal origin of embryonic cell lineages in adult chimeras formed between *Mus musculus* and *Mus caroli* mice (28, 29). However, to take advantage of this marker in characterizing mutant ES cell lines, the latter would need to be derived from inner cell mass of the species *Mus musculus* and chimeras formed with a species such as *Mus caroli*, whose DNA does not significantly cross-hybridize to the satellite DNA probe.

A third advantage of the β-globin transgene as a marker is that it can be detected at extremely high resolution—even higher resolution than the satellite DNA probe as the signal in that case tends to obscure nuclear morphology (29). *In situ*

hybridization has detected the β-globin transgene in 9.5-dpc (Fig. 3) and 8-dpc embryos (18) and it should be observable at even earlier stages of embryogenesis. Tracking of the transgene will also allow the detection of subtle, cell autonomous, phenotypes by mutant ES cells, particularly in heterozygotes or in null mutants that do not give rise to viable mice. In particular, the WW6 line provides an approach to looking at the effects of null mutations on patterns of tissue and cell type colonization. For example, it should be possible to detect genes that lead to the exclusion of mutant ES cells from a certain cell population or to an altered pattern of colonization and genes that give rise to autonomous versus nonautonomous cellular phenotypes; and, finally, it should be possible to look at gene dosage or other effects that give rise to a partial phenotype. The feasibility of this approach is amply demonstrated by the data in Fig. 3 showing that even WW6 cells that have gone through two rounds of gene targeting give rise to strong chimeras. In summary, the overall characteristics of the β-globin transgene in WW ES cells fulfill most of the properties of the ideal marker desired for chimeric studies (26, 30). These criteria include genetic stability, ubiquitous expression in all tissues, developmental inertness, lack of a need for expression, cell localization, and ease of detection.

We thank Dr. Cecilia Lo for the mouse globin gene clone and for helpful advice; Dr. Davor Solter for providing breeding pairs of strain TgN(Hbb-b1)83Clo mice; Dr. Frank Costantini for globin gene cDNA; Dr. Jing Yang for the *Mgat1* gene probe; Jim Horner for 14.5-dpc WW6-derived chimeric embryos; and the Stem Cell Facility at the Albert Einstein College of Medicine for injections of blastocysts. We especially thank Dr. Elizabeth Robertson for critical comments on the manuscript. This work was supported by Grants NCI R37 30645 (P.S.) and HD RO1 25208 (F.P. in the laboratory of E. J. Robertson) and by Training Grant T32 CA09060 (E.I.) from the National Institutes of Health. Support was also provided by Cancer Center Grant PO1 13330 from the National Cancer Institute.

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