Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4712–4716, June 1990
Genetics

Targeted replacement of the homeobox gene *Hox-3.1* by the *Escherichia coli lacZ* in mouse chimeric embryos

(transgenesis/embryonic stem cells/homologous recombination/β-galactosidase)

HERVÉ LE MOUELLIC, YVAN LALLEMAND, AND PHILIPPE BRÛLET*

Unité de Génétique Cellulaire du Collège de France et de l'Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France

Communicated by François Jacob, March 28, 1990

ABSTRACT **Through gene targeting based upon homologous recombination in embryonic stem cells, a chosen gene can be inactivated and eventually a strain of mutant mice created. We have devised a procedure to specifically replace a targeted gene by another gene. A murine homeobox gene was disrupted at high frequency in embryonic stem cells by its replacement with *Escherichia coli lacZ*. Injection of such stem cells into blastocysts yielded chimeric embryos in which β-galactosidase activity was driven by the *Hox-3.1* promoter. This technique will allow the visual assessment at the cellular level of gene inactivation effects in transgenic mice.**

Mice with altered genes can be generated through gene targeting in embryonic stem (ES) cells (1, 2). First, the desired mutation is introduced into the cloned sequences of a given locus. The mutated DNA electroporated into ES cells will integrate, usually at low frequency, into its appropriate genomic location by using the cellular machinery for homologous recombination (3, 4). Germ-line chimeras can be generated by microinjecting recombined ES cells into blastocysts (5–8). The interbreeding of heterozygous siblings will yield animals homozygous for the desired mutation. Such a scheme should allow the detailed analysis of the mutated gene in various genetic backgrounds.

A sophisticated genetic analysis is required to understand the complex biological process of mammalian embryogenesis. We expect that many genes differentially regulated during embryogenesis form networks that underlie morphogenesis. A simple null mutation could lead to a complete disruption of a regulatory network; on the other hand, the differences between mutant and wild-type phenotypes might be minor because of the possible redundancy of genes controlling embryogenesis. Understanding the effects of gene inactivation would be easier if we could follow at the cellular level the eventual appearance of a phenotype induced by a defined mutation, dominant or recessive. Methods used to date to inactivate genes by homologous recombination (2) do not easily allow one to follow the fate of cells that normally would express that gene. We have developed a procedure whereby a chosen gene is not only inactivated but also replaced by the functional reporter gene of *Escherichia coli* for β-galactosidase (*lacZ*). The endogenous promoter of the targeted gene controls the reporter gene, whose expression can be followed *in situ* throughout embryogenesis of the mutant animal.

We have applied this procedure to *Hox-3.1*, a member of the mouse *Hox* gene family, after having previously described its complex pattern of transcript accumulation throughout embryogenesis by *in situ* hybridization analysis (9). Homeobox genes of vertebrates and *Drosophila* share extensive homologies in sequence and organization and are arranged in similar complexes (10). The mouse genome contains four *Hox* complexes, probably having arisen from a single ancestral complex by duplication (10). Analogously positioned murine homeobox genes of different clusters display overlapping, although not identical, transcription patterns during embryogenesis (11). Furthermore, in the neural tube of midgestation embryos, the spatial transcription patterns of linked homeobox genes display respective anterior limits that correlate with their order or arrangement within their clusters (10, 12). These results give circumstantial indications that the vertebrate homeobox genes are involved in the specification of positional information during embryogenesis. The replacement of a murine homeobox gene by *lacZ* should help to establish its function during development. In mice in which both *Hox-3.1* alleles are inactivated by *lacZ* replacement, staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) should reveal the developmental fate of cells that lose *Hox-3.1* expression.

In experiments to inactivate the mouse *Hox-3.1* gene, we obtained a high ratio of homologous recombination versus random integration (1/40). Such high efficiencies were also obtained by using the herpes simplex virus gene for thymidine kinase, *tk*, to negatively select against random integration events (13) or by microinjecting a mutated gene fragment into ES cell nuclei (14). Mutated ES cells were detected by polymerase chain reaction (PCR), cloned, and used to obtain chimeric embryos in which β-galactosidase activity reflected *Hox-3.1* expression.

MATERIALS AND METHODS

Cell Cultures and Transfections. The CCE (5) ES cell line was maintained continuously on feeder layers from primary embryonic fibroblasts (15). In experiments I and II, 1.5×10^7 ES cells in 1.5 ml of HeBS were electroporated (see ref. 16) at 200 V with 40 μg of linearized plasmid and then plated on four 100-mm culture dishes. In experiment III, the pulse was performed under the same conditions, but only one-fourth of the cells were plated on four 24-well plates. G418 was added the next day at 250 μg/ml. Each transfection yielded about 2400 clones with pGMA and about 1000 clones with pGMD.

Individual ES colonies were isolated from positive pools by picking them with pulled pipettes. The isolated clones were from then on cultured on feeder layers with the addition of 1000 units/ml of LIF (17, 18).

PCR and Southern Analyses. DNA from ES clones was prepared at the time of replica plating with a "boiling–proteinase K digestion–boiling" method (19). Forty cycles of amplification (40 sec at 94°C, 1 min at 60°C, and 7 min at 72°C) were performed in a 100-μl reaction mixture [67 mM Tris·HCl, pH 8.6/16.7 mM $(NH_4)_2SO_4$/6.7 mM $MgCl_2$/10

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ES cells, embryonic stem cells; PCR, polymerase chain reaction; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; Neo^r, neomycin resistance; G418^r, G418 resistant; RSV, Rous sarcoma virus, SV40, simian virus 40; p.c., postcoitus.

*To whom reprint requests should be addressed.

mM 2-mercaptoethanol/0.01% gelatin/200 μM dATP/200 μM dTTP/200 μM dCTP/100 μM dGTP/100 μM 7-deaza-dGTP (20)/600 ng of each primer/3 units of *Thermus aquaticus* (*Taq*) polymerase (Perkin–Elmer/Cetus)] covered by 100 μl of paraffin.

Animal and Embryo Manipulation. Blastocysts [3.5-day postcoitus (p.c.) embryos] were obtained (15) from superovulated (BALB/c × SJL/J)F_1 3-week-old females mated with males of the same genotype. Injected blastocysts were introduced into pseudopregnant recipient mice. This was considered to be day 2.5 p.c. (21), the 0.5 day being the day of the plug of the bearer mother. Embryos were collected after implantation and stained for β-galactosidase activity by a 15-min fixation in phosphate-buffered saline (PBS) containing 1% formaldehyde and 0.2% glutaraldehyde, two washes in PBS, and an overnight staining at 37°C in PBS containing 0.4 mg of X-Gal per ml, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6 \cdot 3H_2O$, and 4 mM $MgCl_2$.

RESULTS AND DISCUSSION

Targeted Replacement Vector. The plasmid pGN described in Fig. 1*a* was the basic vector designed to disrupt a given locus by replacing it by the *lacZ* gene. For this purpose, a multiple cloning site was located 5′ from the β-galactosidase reading frame with a consensus eukaryotic translation initiation sequence (23). Genomic sequences providing the DNA homologous to the locus to be specifically replaced can be cloned into the multiple cloning site, while retaining a site for vector linearization. In the case of *Hox-3.1*, homologous recombination between the chromosomal *Hox-3.1* locus and the colinear plasmid sequences (Fig. 1*b*) resulted in the replacement of a coding region of *Hox-3.1* (codons 2–42) by a large insert (7.2 kb). Thus, the *lacZ* gene was put under the influence of transcription regulatory regions of *Hox-3.1* localized upstream from its AUG start codon.

The pGN plasmid and the targeting vectors derived from it contain a selection unit based on the Neo^r gene from the pRSV-neo plasmid (25). However, the Neo^r gene and surrounding sequences were modified to achieve more efficient selection of G418^r ES clones while retaining the use of bacteria selection with kanamycin. Depending on the experimental conditions, homologous recombination can be a rather rare event (e.g., 1/1000 for *HPRT*; ref. 4). Thus, a vector with a high selection efficiency might be useful, especially as the electroporation conditions used have been shown to mediate mainly single-copy integrations (26). A resistance gene with a strong promoter in ES cells might be less influenced by the site of vector integration and thus give access to homologous recombination in a larger part of the genome. Furthermore, the enhanced resistance of ES clones should decrease the selective pressure and thus favor their growth. With the addition of mutant polyoma enhancers and the modification of the translation initiation sequence of the Neo^r gene as shown in Fig. 1*a*, the pGN vector yielded, at equimolar amounts, 450 times more G418^r clones compared with pRSV-neo. We also compared pGN to a similar selection unit, the pMC1-neo plasmid of Thomas and Capecchi (4),

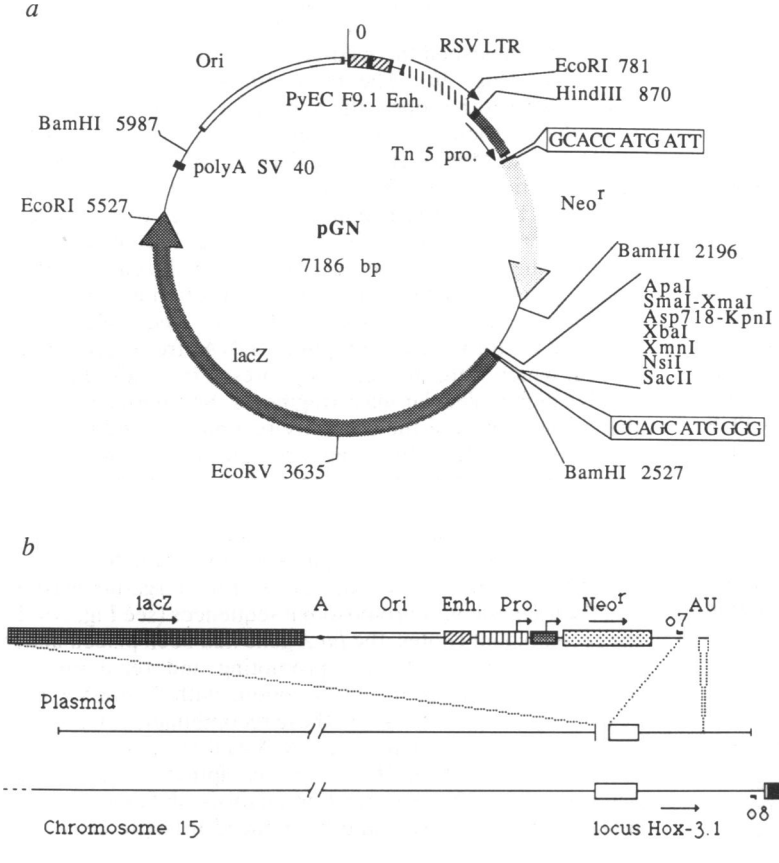

FIG. 1. (*a*) Cloning vector pGN. The neomycin resistance (Neo^r) transcription unit begins with a tandem direct repeat of the enhancer (Enh.) unit B of the polyoma virus (a *Pvu* II–*Pvu* II fragment from the PyEC F9.1 strain) in the late mRNA orientation (22). When compared with pRSV-neo, the number of G418 resistant (G418^r) ES clones was thus increased 7.5 times with these enhancers. A eukaryotic promoter, the Rous sarcoma virus (RSV) long terminal repeat (LTR), and a prokaryotic promoter (Pro.) from Tn*5* direct the transcription of the phosphotranferase gene encoding Neo^r. The 5′ sequence flanking the Neo^r AUG initiation codon was modified by means of oligonucleotide-directed mutagenesis on a single-strand template. TCGC*ATG* was turned into the vertebrate consensus sequence (23), CACC*ATG*, to raise the translation initiation efficiency in mammalian cells. The number of G418^r ES clones was again increased 60 times, resulting in a 450-fold improvement when compared with pRSV-neo. With this modified translation initiation codon, the resistance of bacteria was reduced but still usable at the selective kanamycin concentration of 20 $\mu g \cdot ml^{-1}$. *lacZ* represents the coding sequence of the *E. coli* β-galactosidase gene in phase with the AUG codon from *Hox-3.1* and is followed by the simian virus 40 (SV40) polyadenylylation and transcription termination signals. A multiple cloning site has been inserted 5′ of the *lacZ* gene. Ori is the ColE1 origin of replication. (*b*) *Hox-3.1*-targeted replacement vector pGMD. The two parts of the coding sequence of the *Hox-3.1* locus (9), located on chromosome 15 and separated by an intron, are represented by rectangles, with the homeobox darkened. The mutagenesis plasmid was linearized by *Nsi* I digestion prior to electroporation of ES cells. Its ends are then the two genomic arms that were cloned into the *Apa* I–*Nsi* I and *Nsi* I–*Sac* II sites of pGN. The 5′ and 3′ arms, according to the transcription orientation of *Hox-3.1*, are 6.8 and 1.5 kilobases (kb) long, respectively. In pGMD the Neo^r gene is not followed by a polyadenylylation signal but instead by an A+U-rich sequence responsible for a selective mRNA degradation (24), inserted in the *Hox-3.1* intron sequence of the plasmid. o7 (AACTTCCCTCTCTGCTATTC) and o8 (CAGCAGAAACATACAAGCTG) are the oligonucleotides used for PCR. Arrows indicate transcription/translation orientations. Another targeted replacement vector, pGMA, was identical to pGMD except that it contained SV40 polyadenylylation and transcription termination signals but no A+U-rich mRNA degradation sequence 3′ to the Neo^r gene. bp, Base pairs.

which differs from the pGN selection unit by the herpes simplex virus *tk* promoter, the translation initiation sequence, and no bacterial promoter. In our experimental conditions, pGN yielded 2 to 3 times more clones than pMC1-neo.

In the event of homologous recombination, the Neo^r gene will be followed by the intron and the polyadenylylation and transcription-termination signals of *Hox-3.1*. The SV40 mRNA 3′ processing signals were left downstream of the Neo^r gene of a targeting vector, pGMA, but were removed in a second one, pGMD (Fig. 1*b*), to test an enrichment procedure for homologous recombination events. In addition, an A+T sequence shown to be responsible for selective cytoplasmic degradation of mRNA in different cell lines (24) was inserted into the *Hox-3.1* intron sequences of pGMD (Fig. 1*b*). These modifications were intended to lower the level of Neo^r transcripts in clones resulting from random integration events. We reasoned that the number of such clones should be reduced, or at least their growth should be slowed down. On the other hand, clones issued from homologous recombination between pGMD and a *Hox-3.1* locus should have an unaltered growth during G418 selection, the mRNA degradation A+T sequence being eliminated by the recombination process itself or spliced out with the *Hox-3.1* intron.

***Hox-3.1* Inactivation in ES Cells.** Initial transfections were performed as described (16) to compare the efficiencies of *Hox-3.1* targeting with the pGMA and pGMD plasmids in CCE ES cells (5) (Table 1). For this purpose a sensitive and rapid assay for homologous recombination was based upon the PCR (19). Only in the case of homologous recombination event was a 1.6-kb fragment amplified (Fig. 2) by using one primer, o7, homologous to sequences on the cloning vector, and another one, o8, corresponding to 3′ flanking *Hox-3.1* sequences (see Fig. 1*b*). Specific amplification was confirmed by Southern hybridization with an appropriate *Hox-3.1* probe not containing the PCR primer sequences.

From the initial analysis of pools of 250–600 clones (Table 1), we estimated a homologous recombination frequency higher than 1/250 with pGMD and of about 1/900 with pGMA. Other transfections with pGMD were performed so as to minimize the cumbersome cloning of mutated ES cells and allow a better estimate of the ratio of homologous recombination to random insertion. After electroporation, cells were plated at low density so that only a few clones per well would grow (Table 1, experiment III). DNAs from ES cells in several wells were pooled and subjected to PCR analysis (Fig. 2). Individual wells were subsequently analyzed. Five independent clones yielded positive PCR signals. The ratio of homologous recombination versus random integration was then 1/40 with a pGMD plasmid, as estimated from the total number of 210 G418^r clones. For two of those clones, we could show the presence of the A+T sequence on the 1.6-kb amplified fragment, indicating that recombination did occur between the A+T sequence and the 3′ end of the

Table 1. Homologous recombination into *Hox-3.1*

Exp.	Targeting vector	Pools analyzed	Size of pools* (no. of clones)	Positive PCR results,† no.
I	pGMA	3	600	0 (2)
II	pGMD	5	250	3 (5)
III	pGMD	84	2–3	5 (5)

*The average number of G418^r ES clones in analyzed pools was estimated by counting one independent fourth of the clones of each transfection.

†Positive PCR results are shown on Fig. 2. In parentheses is indicated the number of transfection pools showing a positive signal after Southern blotting of the PCR and hybridization to a specific probe—i.e., not overlapping the oligonucleotide sequences (probe *a* in Fig. 3*c*).

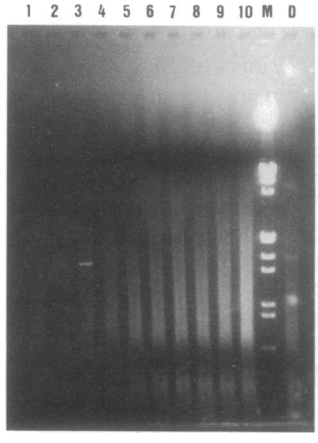

FIG. 2. Detection of homologous recombination with PCR in ES transfection pools. Lanes: D, PCR performed on 10^5 cells from a pool of about 250 clones from transfection II (see Table 1); 1–10, four pools from transfection III, of 2 or 3 clones each, were analyzed together by mixing ≈4 × 5000 cells (half of the reaction was run in a 0.7% agarose gel stained with ethidium bromide); M, the size marker is an *Eco*RI/*Hin*dIII digest of phage λ DNA. Lanes 2, 3, and D show a 1.6-kb-long amplified fragment.

homologous DNA. The other three clones had lost the A+T sequence in homologous recombination events, which presumably have occurred between the insert of the targeting vector and the A+T sequence (see Fig. 1*b*).

Three independent *Hox-3.1*-mutated ES cell clones were isolated. Their DNAs were examined with Southern blotting after digestion with several restriction enzymes to confirm specific targeting and to allow the distinction between the recombined and the wild-type loci. Two different probes were used to analyze the 3′ end of the *Hox-3.1* loci in the mutated clones and in unmutated ES cell controls (Fig. 3*c*). The first probe (*a*) displayed the number of vector integrations and their physical linkages. One of the three recombined clones contained in addition a randomly integrated plasmid copy (Fig. 3*a*, clone F2). The second probe (*b*) clearly distinguished the wild-type and the recombined *Hox-3.1* alleles (Fig. 3*b*). The recombined *Hox-3.1* locus displayed, with both probes, the hybridization pattern expected from the restriction maps of the targeting vector and the intact locus. Moreover, the existence of two domains of recombination in the 3′ arm of the targeting vector was confirmed by the presence or absence of the A+T sequence in the recombined *Hox-3.1* locus (e.g., Fig. 3, clone L5). The 5′ end of the *Hox-3.1* locus was similarly analyzed for correct homologous recombination event. Restriction enzymes that do not cleave in the 6.8-kb 5′ *Hox-3.1* sequence of the targeting vector were used to digest DNAs from recombined clones, which were then electrophoresed in a pulsed field to resolve high molecular mass fragments. Southern analysis of this gel also showed the correctly recombined and the wild-type *Hox-3.1* alleles by using a 5′-end probe sequence upstream from the targeting plasmid (data not shown).

The Southern analyses demonstrated that one allele of the *Hox-3.1* gene had been recombined as expected. The homologous recombination was equivalent to a double crossing over between the two genomic arms of the targeting plasmid and the homologous chromosomal sequences (see Fig. 1*b*). In the recombinant clones, the *lacZ* gene had been placed under the control of the *Hox-3.1* promoting and regulating sequences upstream of its AUG codon, with 3′ mRNA processing signals from SV40. In these recombinant clones, *lacZ* expression was not detectable by X-Gal staining, in agreement with the lack of *Hox-3.1* transcription in ES cells as determined by RNase protection analysis (data not shown). β-Galactidase activity could be induced in some cells after 3–4 days of culture in the presence of 0.5 μM retinoic acid (data not shown), conditions known to induce *Hox-3.1* transcription in EC cells (27).

By using the pGMA targeting vector with an 8.3-kb DNA overall homology to the *Hox-3.1* locus, a 120-bp genomic fragment was replaced by a 7.2-kb insert. The frequency of this targeted replacement (1/900) was comparable to recent reports of homologous recombination [1/1000 for *HPRT* (4), or

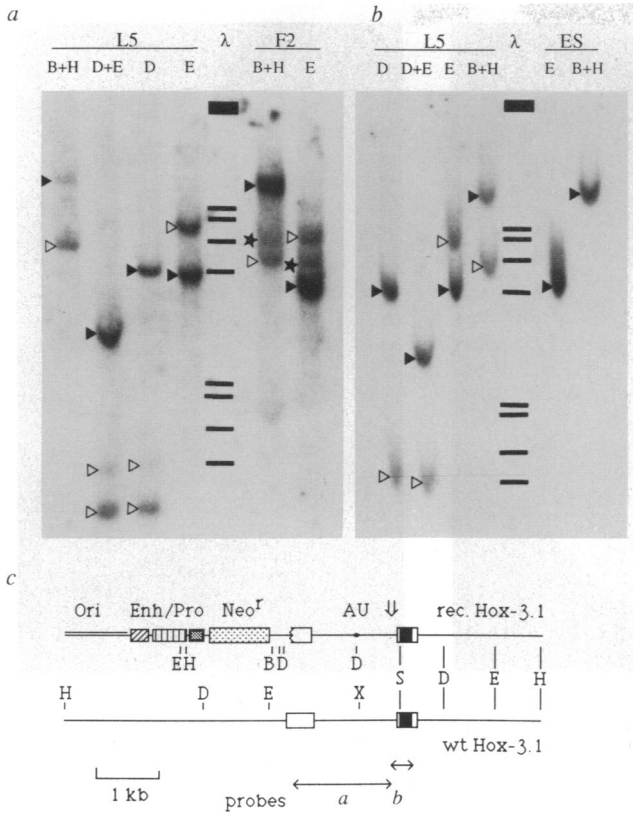

FIG. 3. (*a* and *b*) Southern analyses of individual positive clones (L5 and F2) and control ES cells (CCE). The probes used hybridize only to *Hox-3.1* sequences included in (*a*) or excluded from (*b*) the targeting vector. The hybridization pattern of the recombined *Hox-3.1* locus (open arrowheads) was clearly distinguishable from the wild-type locus (filled arrowheads). Stars point to hybridizing bands of a randomly integrated plasmid copy. Each lane contains 12 μg of digested genomic DNA. The size marker is an *Eco*RI/*Hin*dIII digest of phage λ DNA. (*c*) Restriction maps of the recombined (rec.) and wild-type (wt) *Hox-3.1* alleles. Parts of the targeting vector and the *Hox-3.1* locus are indicated as in Fig. 1. In this case, the A+T sequence was integrated by the homologous recombination event. The vertical arrow points to the 3′ end of the targeting vector. Probes *a* and *b* used in Southern analyses are also indicated. B, *Bam*HI; D, *Dra* I; E, *Eco*RI; H, *Hin*dIII; S, *Sal* I; X, *Xho* I.

1/260 for *En-2* (28)] where the heterologous insert was however much smaller (1.1 and 1.5 kb, respectively). An unexpected finding was the high rate of homologous recombination when using the pGMD plasmid (1/40). Removal of the 3′ mRNA processing signals and addition of a mRNA degradation sequence in the *Hox-3.1* intron did not prevent the Neo^r gene from working in numerous clones resulting from random integration. The overall number of G418^r ES clones obtained with pGMD was decreased only by a factor of 2.4 as compared with pGMA. However, the ratio of specific targeting was nearly 10-fold higher (900/40), suggesting that the mechanism of homologous recombination itself may have been affected in experiments with pGMD. Recently, a comparable ratio of homologous recombination in the β_2-microglobulin gene was potentially explained by the possible existence of a hotspot of recombination in this locus and the lack of polyadenylylation site to the selection gene (8). A possible explanation of our data is that a 51-bp A+T sequence could provide *in vivo* an opening loop in the targeting plasmid because of its lower melting temperature. If the neighboring pGMD *Hox-3.1* sequences on both sides of the A+T region can be influenced by this opening, they could interact more efficiently, when single-stranded, with the chromosomal *Hox-3.1* locus. A model of mitotic recombination in yeast (29) suggests that it would be initiated by such a strand exchange, although the mechanism of the homologous recombination is still unknown in higher eukaryotic cells.

***lacZ* Expression in Chimeric Embryos.** Microinjections of ES cells into blastocysts were done by using two recombinant ES clones that contained an intact and a recombined *Hox-3.1* allele and no other copy of the targeting vector. Karyotypes of these cells were normal. Ten to 15 mutated cells were microinjected into each blastocyst. After reimplantation into recipient mothers, embryos were collected at days 9.5, 10.5, and 12.5 p.c. and were analyzed for *lacZ* expression (32). The *Hox-3.1* expression pattern at these stages has been established by *in situ* hybridization analysis (9). When first detectable at late gastrulation stages, Hox-3.1 transcripts are distributed in all of the tissues of the posterior end. At later stages, the distribution becomes progressively spatially restricted and tissue specific. By 12.5 days p.c., transcription is localized to the cervical region of the neural tube, at the level of the heart. Thus, during the course of embryogenesis, the *Hox-3.1* transcription pattern undergoes gradual modifications. Day 10.5 p.c. appears to be a transition period displaying transcription in both posterior regions and the cervical neural tube.

In 9.5- and 10.5-day p.c. chimeric embryos, the caudal region posterior to the hindlimb bud displayed intense β-galactosidase activity, whereas no labeling was ever detected in the anterior thoracic region or the head (Fig. 4*a*). In the posterior region, X-Gal-stained cells were observed (on paraffin sections) in tissues from all germ layers—i.e., in the mesenchyme, the hindgut, the neural tube, the neural crest, and the skin (data not shown). Between the two limb buds, X-Gal-labeled cells were distributed in small patches in the overlying ectoderm (Fig. 4*b*) and posterior regions (Fig. 4*c*) and in thin lines in the dorsal wall of the neural tube (Fig. 4*b*). These stripes displayed an irregular and nonsymmetrical distribution in the dorsal wall of the neural tube. *Hox-3.1* transcription was not previously detected in such a thin sheet of cells at the closure of the neural tube, which may not have withstood the treatments used in *in situ* hybridization. Our observations support the findings of others that cells of the neural ectoderm are committed early to different parts of the nervous system and migrate in a radial direction following very narrow lateral paths (30).

Thus, *lacZ* expression correctly reflected the first component of the homeobox gene *Hox-3.1* transcription—i.e., in all tissues of 9.5- and 10.5-day p.c. embryos' caudal regions—and provided new information on the *Hox-3.1* transcription pattern not seen before by *in situ* hybridization. In contrast, no labeling was observed in the cervical regions of the neural tube in 12.5-day chimeric embryos or in the anterior region of transcription in 10.5-day embryos, as would have been expected from *in situ* hybridization data. Further experiments will be needed to understand the reasons for the differences in the results of our experimental techniques.

CONCLUSION

We have shown that a specific gene replacement can be mediated by the recombination machinery of ES cells. Targeted replacement resulted in the inactivation of a target gene by the introduction of a marker gene. The expression of the newly introduced gene was then regulated by the promoter of the mutated gene. In our experiments, neither the size of the insert (7.2 kb) nor the lack of transcription of the target locus in ES cells prevented a high frequency of homologous recombination (1/40).

Since *lacZ* expression is easily detected *in situ* at the level of just a few cells, this replacement technique should allow a more detailed expression analysis than *in situ* hybridization. An example of such analysis with its advantages and its

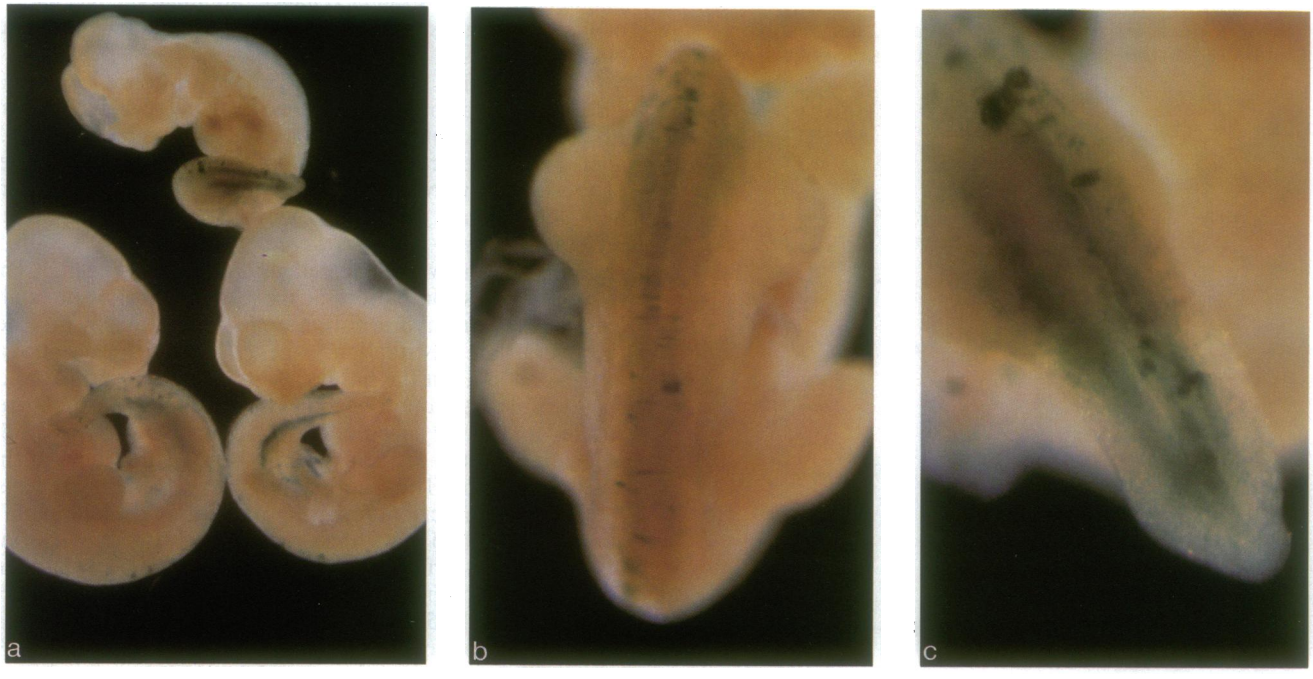

FIG. 4. *lacZ* expression in 10.5-day p.c. chimeric embryos. (*a*) Three chimeric embryos obtained by injection of the L5 recombined ES clone. The upper embryo had a delayed development and an abnormality in the closure of its head. Such abnormalities occurred also in litters of unmanipulated embryos. (*b*) Dorsal view, between the limb buds, of the lower left embryo in *a*. The head is on the top, in the background. (*c*) Caudal part of the upper embryo in *a*.

limitations has been presented for the *Hox-3.1* homeobox gene. As the fate of the cells that normally expressed *Hox-3.1* could be followed, distribution of clones during development could be observed in chimeras, as suggested by the thin stripes of β-galactosidase activity in part of the neural tube. In conjunction with transplantation experiments, such replacement technology could provide a new tool to correlate a specific gene promoter activity and cellular determination.

Targeted gene replacement has different applications, depending upon the reporter and the targeted genes. Cell lineage ablation (31) or specific immortalization of precursor cells could be attempted with reporter genes encoding toxins or conditional oncogenes, respectively (see ref. 30). As shown here, targeted gene replacement with an *in situ* marker gene will be particularly useful to follow at the cellular level the appearance of a phenotype throughout embryogenesis of heterozygous and especially homozygous mutant mice.

We thank Martin Evans for the gift of the CCE ES cell line, Jean-François Moreau for providing us with leukemia inhibitory factor, and Tom Hollon for critically reading the manuscript. This work was supported by grants from the Centre National de la Recherche Scientifique (URA 1148), the Ligue Nationale Française Contre le Cancer, the Association pour la Recherche sur le Cancer (6609), and the Direction des Recherches et Etudes Techniques (86165).

1. Capecchi, M. R. (1989) *Trends Genet.* **5,** 70–76.
2. Rossant, J. & Joyner, A. L. (1989) *Trends Genet.* **5,** 277–283.
3. Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A. & Kucherlapati, R. S. (1985) *Nature (London)* **317,** 230–234.
4. Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51,** 503–512.
5. Robertson, E., Bradley, A., Kuehn, M. & Evans, M. (1986) *Nature (London)* **323,** 445–448.
6. Gossler, A., Doetschman, T., Korn, R., Serfling, E. & Kemler, R. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9065–9069.
7. Thompson, S., Clarke, A. R., Pow, A. M., Hooper, M. L. & Melton, D. W. (1989) *Cell* **56,** 313–321.
8. Zijlstra, M., Li, E., Sajjadi, F., Subramani, S. & Jaenisch, R. (1989) *Nature (London)* **342,** 435–438.
9. Le Mouellic, H., Condamine, H. & Brûlet, P. (1988) *Genes Dev.* **2,** 125–135.
10. Akam, M. (1989) *Cell* **57,** 347–349.
11. Gaunt, S. J., Krumlauf, R. & Duboule, D. (1989) *Development Suppl.* **107,** 131–141.
12. Wilkinson, D. G., Bhatt, S., Cook, M., Boncinelli, E. & Krumlauf, R. (1989) *Nature (London)* **341,** 405–409.
13. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. (1988) *Nature (London)* **336,** 348–352.
14. Zimmer, A. & Gruss, P. (1989) *Nature (London)* **338,** 150–153.
15. Robertson, E. J. (1987) in *Teratocarcinomas and Embryonic Stem Cells,* ed. Robertson, E. J. (IRL, Oxford), pp. 71–112.
16. Chu, G., Hayakawa, H. & Berg, P. (1987) *Nucleic Acids Res.* **15,** 1311–1326.
17. Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A. & Gough, N. M. (1988) *Nature (London)* **336,** 684–687.
18. Smith, A. G., Heath, J. K., Donaldson, D. D., Wong, G. G., Moreau, J., Stahl, M. & Rogers, D. (1988) *Nature (London)* **336,** 688–690.
19. Kim, H.-S. & Smithies, O. (1988) *Nucleic Acids Res.* **16,** 8887–8903.
20. McConlogue, L., Brow, M.-A. D. & Innis, M. A. (1988) *Nucleic Acids Res.* **16,** 9869.
21. Bradley, A. (1987) in *Teratocarcinomas and Embryonic Stem Cells,* ed. Robertson, E. J. (IRL, Oxford), pp. 113–151.
22. Herbomel, P., Bourachot, B. & Yaniv, M. (1984) *Cell* **39,** 653–662.
23. Cavener, D. R. (1987) *Nucleic Acids Res.* **15,** 1353–1361.
24. Shaw, G. & Kamen, R. (1986) *Cell* **46,** 659–667.
25. Gorman, C., Padmanabhan, R. & Howard, B. H. (1983) *Science* **221,** 551–553.
26. Boggs, S. S., Gregg, R. G., Borenstein, N. & Smithies, O. (1986) *Exp. Hematol.* **14,** 988–994.
27. Brier, G., Bucan, M., Francke, U., Colberg-Poley, A. M. & Gruss, P. (1986) *EMBO J.* **5,** 2209–2215.
28. Joyner, A. L., Skarnes, W. C. & Rossant, J. (1989) *Nature (London)* **338,** 153–156.
29. Szostak, J. W., Orr-Weaver, T. L. & Rothstein, R. J. (1983) *Cell* **33,** 25–35.
30. McKay, R. D. G. (1989) *Cell* **58,** 815–821.
31. Palmiter, R. D., Behringer, R. R., Quaife, C. J., Maxwell, F., Maxwell, I. H. & Brinster, R. L. (1987) *Cell* **50,** 435–443.
32. Sanes, J. R., Rubenstein, J. L. & Nicolas, J. F. (1986) *EMBO J.* **5,** 3133–3142.