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Low Level of Hox1.3 Gene Expression Does Not Preclude the Use of Promoterless Vectors To Generate a Targeted Gene Disruption†

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A variety of experimental approaches have been devised recently to mutate mammalian genes by homologous recombination. In this report, we describe the disruption of the Hox1.3 locus by using two of these approaches, namely, positive-negative selection and activation of a promoterless gene. Interestingly, we observe similarly high frequencies of targeted disruption with both procedures. The high frequency of targeted disruption with a promoterless vector was unexpected given the extremely low level of Hox1.3 expression in the embryonic stem cell line used for these studies. These data indicate that minimal expression of the target gene is required to enrich for homologous recombination events with promoterless vectors and thus enhance the promoterless gene approach as a general strategy to mutate mammalian genes by homologous recombination.

Gene targeting, by means of homologous recombination between exogenous DNA and endogenous genomic sequences, allows the generation of specifically designed mutations in mammalian cells (for a review, see reference 6). In combination with the embryonic stem (ES) cell technology (for a review, see reference 32), it is now possible to generate strains of mice carrying predetermined mutations (9, 19, 21, 27, 35, 39, 40, 43, 44, 47).

A major limitation in the gene-targeting approach is that DNA introduced into mammalian cells integrates by random, illegitimate recombination approximately 100 to 100,000 times more frequently than by homologous recombination. In order to preferentially recover the small fraction of cells in which a homologous recombination event occurs, two procedures, positive-negative selection (PNS) (25) and activation of a promoterless gene (7, 10, 17, 36, 37), have been devised. The PNS approach requires the inclusion of two selectable markers in the targeting vector. The first marker (e.g., *neo*, which confers G418 resistance) is embedded in the sequences of homology and is used to select for cells that have incorporated the vector. The second marker (e.g., herpes simplex virus thymidine kinase [HSV-*tk*], which confers sensitivity to purine analogs such as ganciclovir [GANC], FIAU, and acyclovir) is placed outside of the regions of homology. As a consequence of integration via double reciprocal crossover at the target locus, the HSV-*tk* marker will be deleted from the targeting vector. Thus, enrichment for cells which have undergone homologous recombination is achieved by selecting for G418- and GANC-resistant cells (i.e., neo^+, HSV-tk^-). One postulated advantage of PNS replacement vectors is that there is no requirement for expression of the target gene. In contrast, the detection of a homologous recombination event by the second approach, namely, the activation of a promoterless targeting vector, is in principle contingent on integration into an active transcriptional unit. Thus, expression of the selectable gene will only occur either as a consequence of random integration into an active transcriptional region or after integration by homologous recombination into the target gene. Since activation by cellular promoters occurs in approximately 1% of random integration events, the use of promoterless vectors provides a 100-fold enrichment for homologous recombination by reducing the background of nonhomologous integrations (17, 37).

The mouse genome contains more than 30 genes harboring a homeobox sequence related to that present in *Drosophila melanogaster* homeotic genes (for a review, see reference 20). These Hox genes are organized primarily in four major complexes that have arisen via duplication of an ancestral homeobox gene cluster (34). Analysis of the expression of individual Hox genes in the developing mouse embryo reveals that they are expressed in a restricted domain along the anteroposterior axis. This observation has led to the suggestion that Hox genes are involved in the specification of positional information during embryogenesis (16). Homeobox-containing genes of vertebrates and *D. melanogaster* share extensive homology in their homeodomain sequences and in their relative domains of expression with respect to their structural positions in the genomic complexes (12–14). This evolutionary conservation raises the question of whether the function of the proteins has been similarly conserved. Interestingly, recent studies show that mammalian Hox proteins, when expressed in *D. melanogaster*, can cause the same developmental transformation as their *D. melanogaster* counterparts (23, 26). Definitive evidence for a role for Hox genes in vertebrate development awaits the identification of mutations in Hox genes that perturb normal developmental processes. Recently, the phenotypic analysis of mice homozygous for a targeted gene disruption at the Hox1.5 locus has revealed regionally restricted developmental defects (8).

To begin to study the functional role of the murine Hox1.3 gene during embryogenesis, we generated ES cell clones in which one of the Hox1.3 alleles has been disrupted by homologous recombination by using different strategies of gene targeting. Here we show that while Hox1.3 gene expression in ES cells can only be detected after polymerase chain reaction (PCR) amplification of poly(A)$^+$-selected RNA, Hox1.3 gene disruption with both PNS and promoterless replacement vectors occurs at similarly high frequencies. These data indicate that target genes need not be highly expressed in order to achieve high frequencies of gene

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† This paper is dedicated to Chi Nguyen-Huu.

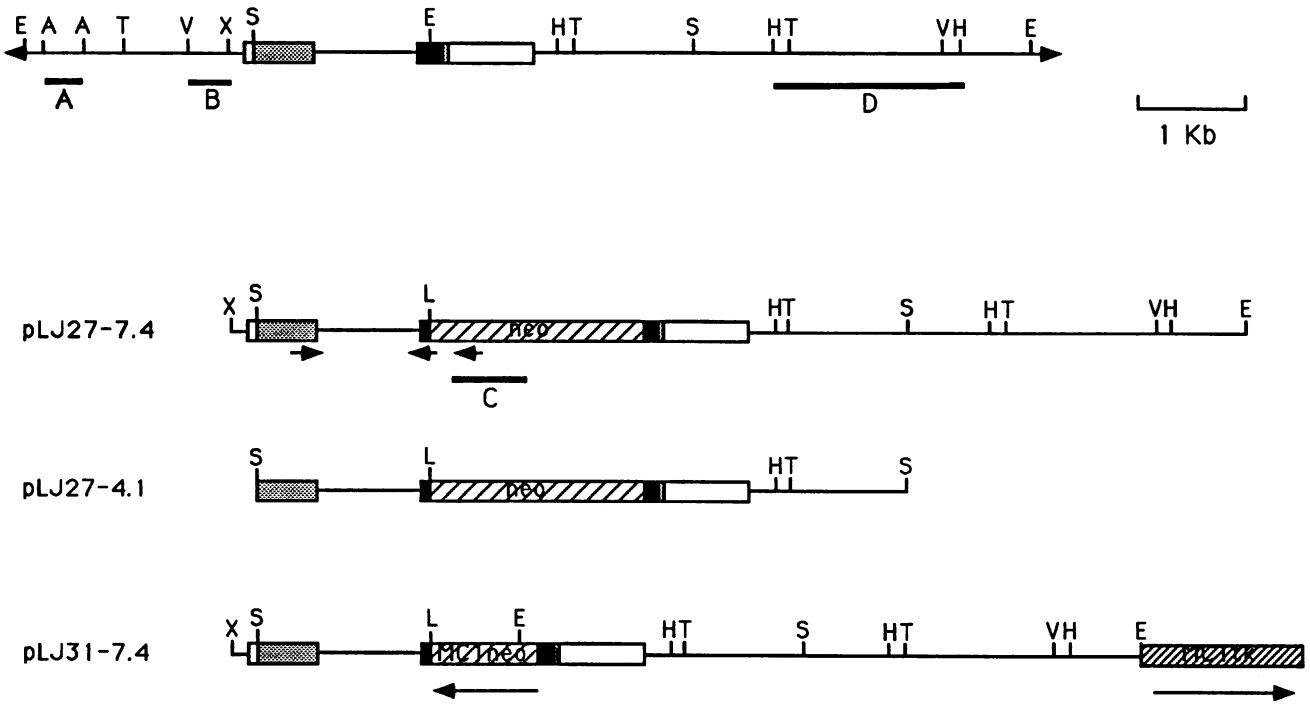

FIG. 1. Schematic diagrams of the Hox1.3 targeting vectors. The structure of the Hox1.3 gene with its two exons is represented above. The coding sequences are shown as stippled boxes, noncoding sequences are shown as open boxes, and the homeobox is shown as a black box. Both targeting fragments pLJ27-7.4 and pLJ27-4.1 contain a promoterless 2-kb *neo* gene inserted in frame into the homeodomain coding sequences. pLJ31-7.4 contains the MC1neoA$^+$ cassette in the homeobox sequence and the MC1-*tk* cassette at the 3′ end of the homology. The *neo* and *tk* sequences are represented by cross-hatched boxes. The probes used for Southern analysis are represented by the thick lines A, B, C, and D. The primers used in the PCR analysis of the Hox1.3 and Hox1.3-*neo* fusion transcripts are represented by the arrows underneath pLJ27-7.4. Abbreviations: A, *Acc*I; E, *Eco*RI; H, *Hin*dIII; L, *Sal*I; S, *Sac*I; T, *Stu*I; V, *Eco*RV; X, *Xba*I.

disruption by using strategies dependent on the activation of a promoterless *neo* gene. This flexibility in targeting approaches will be of value in the design of targeting vectors for genes expressed at similar levels.

MATERIALS AND METHODS

Plasmid constructions. Hox1.3 genomic sequences used for the targeting vectors were derived from a previously described recombinant lambda phage (46). Plasmids pLJ27 and pLJ31 were the source of the targeting DNA fragments used in the promoterless and the PNS approaches, respectively (Fig. 1). In both cases, an 8.8-kb *Acc*I-*Eco*RI genomic fragment containing both exons of the mouse Hox1.3 gene, 1.5 kb of upstream sequences, as well as 4.7 kb of downstream sequences, was subcloned into a pUC18 vector. The *Eco*RI site contained in the homeobox sequence was replaced by a *Sal*I site into which *neo* sequences were inserted. In the case of pLJ27, a transcriptional and translational deficient *neo* gene was inserted in frame into the Hox1.3 coding sequences. A 2-kb *Eco*RV-*Xho*I *neo* fragment containing a simian virus 40 large T antigen poly(A) signal at its 3′ end was excised from pXVX (generously provided by G. Gaitanaris, Columbia University) and inserted in frame into the Hox1.3 homeobox sequence after ligation of *Sal*I linkers to the *Eco*RV site. For pLJ31, the 1-kb *Sal*I-*Xho*I MC1neoA$^+$ cassette (42) was inserted at the same position in the homeobox sequence. In addition, a 1.9-kb *Xho*I-*Hin*dIII pMC1HSV-*tk* fragment (25) was filled in and inserted into a filled-in *Eco*RI site located at the 3′ end of the Hox1.3 sequences. The DNA fragments used in the gene targeting experiments were plasmid pLJ27 DNA digested with *Sac*I (length of genomic homology, 4.1 kb), plasmid pLJ27 DNA digested with *Xba*I and *Eco*RI (length of genomic homology, 7.4 kb), and plasmid pLJ31 DNA digested with *Xba*I (length of genomic homology, 7.4 kb).

Cell culture and electroporation. CCE-ES cells (33) were maintained on mitomycin-treated STOneo^r fibroblast feeder cells as previously described (31). Electroporations were performed under the following conditions. Cells were collected by trypsinization, washed once with electroporation buffer (EB; 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) and resuspended in EB at a concentration of 4×10^7/ml. Cell suspension (0.5 ml) was mixed with 25 μg of the appropriate plasmid DNA. Electroporation was carried out by using a Bio-Rad Gene Pulser apparatus at 200 V and 960 μF. Under these conditions, cell survival was approximately 50%. Cells were plated onto 10-cm feeder plates (approximately 2.5×10^6 surviving cells per plate). Selection was applied 24 h after electroporation with medium containing G418 (GIBCO) at 200 μg of active ingredient per ml for the experiments with pLJ27 fragments or with G418 at 200 μg/ml and GANC (Syntex Research Co.) at 2×10^{-6} M for the experiments with pLJ31 fragment. Cells were refed with fresh drug-containing medium every 2 to 3 days. After 9 to 12 days under selection, individual colonies were picked into 24-well trays for expansion, freezing, and isolation of high-molecular-weight DNA (15). Prior

to DNA extraction, ES cells were grown for two passages on gelatinized plates in the absence of feeder cells.

Identification of homologous recombinant clones. DNA isolated from individual ES cell clones and the parental CCE-ES cell line was first digested with *Eco*RI or *Eco*RV. Approximately 10 μg of the digest was fractionated on a 0.8% agarose gel and transferred to GeneScreen Plus (DuPont). For the *Eco*RI digest, a 400-bp *Acc*I-*Acc*I ^{32}P-labeled probe located outside the targeting fragments (probe A in Fig. 1) was used for hybridization, while for the *Eco*RV digest, a 416-bp *Eco*RV-*Xba*I ^{32}P-labeled probe located outside the targeting fragments (probe B in Fig. 1) was used. In addition, blots were hybridized with a *neo* probe (probe C in Fig. 1), as well as with a pUC18 probe. To ensure that no major rearrangements had occurred in the 5′ and 3′ flanking sequences, DNA from targeted ES cell lines was also digested with *Xba*I, *Eco*RV, or *Stu*I and hybridized with a 5′-specific *Acc*I-*Acc*I probe (probe A) or with a 3′-specific 1.4-kb *Hin*dIII-*Hin*dIII probe (probe D in Fig. 1).

Northern (RNA) blot and PCR analyses of RNA. For RNA extraction, ES cells were grown for two passages on gelatinized plates in the absence of feeder cells. Total RNA from 12.5-day-old mouse embryos, STOneo^r cells, parental CCE-ES cells, and targeted ES cell lines was isolated by the LiCl-urea procedure (3). Poly(A)$^+$ RNA was prepared from total RNA by one-step chromatography on oligo(dT)-cellulose (4). Poly(A)$^+$-selected RNA was fractionated by electrophoresis through a 1% formaldehyde agarose gel (24), transferred to Hybond membrane (Amersham), and hybridized with a 521-bp *Sac*I-*Bgl*II ^{32}P-labeled probe derived from the first exon of the Hox1.3 gene. This probe can detect Hox1.3 mRNA as well as the Hox1.3-*neo* fusion transcript. The blots were also hybridized with a mouse α-actin probe (28), as well as with a mouse dihydrofolate reductase cDNA fragment (41).

For detection of Hox1.3 and Hox1.3-*neo* fusion transcripts with PCR, 2 μg of poly(A)$^+$-selected RNA from the CCE-ES and two targeted ES cell lines as well as 1 μg of poly(A)$^+$ from STOneo^r cells was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) by using the manufacturer's recommended reaction conditions. The reaction mix was denatured at 95°C for 5 min, and reverse transcription reaction was repeated once more. Of the resulting cDNA preparation, 1/10 was then used to amplify either Hox1.3- or Hox1.3-*neo*-specific fragments by PCR. The Hox1.3-specific oligonucleotide primers were 5′TACGGCTACAATGGCAT GGATCTCAGCGTC3′, located in exon 1, and 5′ACAATA TAGGTGGCCCACAAGGCAAAAGGG3′, located in exon 2 (Fig. 1). After PCR amplification, a 453-bp fragment is generated (amplification of the genomic DNA produces a 1.4-kb fragment). For PCR amplification of the Hox1.3-*neo* fusion cDNA, the exon 1 Hox1.3 oligonucleotide was used as a primer along with a *neo*-specific primer, 5′GTGTTCCG GCTGTCAGCGCA3′, located near the 5′ end of the *neo* gene. After PCR amplification, a 645-bp fragment which contains both Hox1.3 and *neo* sequences will be generated. PCR conditions were as follows: 94°C for 2 min, 54°C for 2 min, and 72°C for 3 min repeated for 32 cycles and then 72°C for an additional 5 min in a solution containing 10 mM KCl, 10 mM Tris (pH 8.3), 2 mM dithiothreitol, 1 mM $MgCl_2$, and 0.001% gelatin with 2.5 U of *Taq* DNA polymerase (Perkin-Elmer). One-tenth of the Hox1.3 PCR mixture and one-fifth of the Hox1.3-*neo* PCR mixture were fractionated on a 1.5% agarose gel, transferred to GeneScreen Plus (DuPont), and hybridized with the 521-bp *Sac*I-*Bgl*II ^{32}P-labeled probe derived from the first exon of Hox1.3 gene.

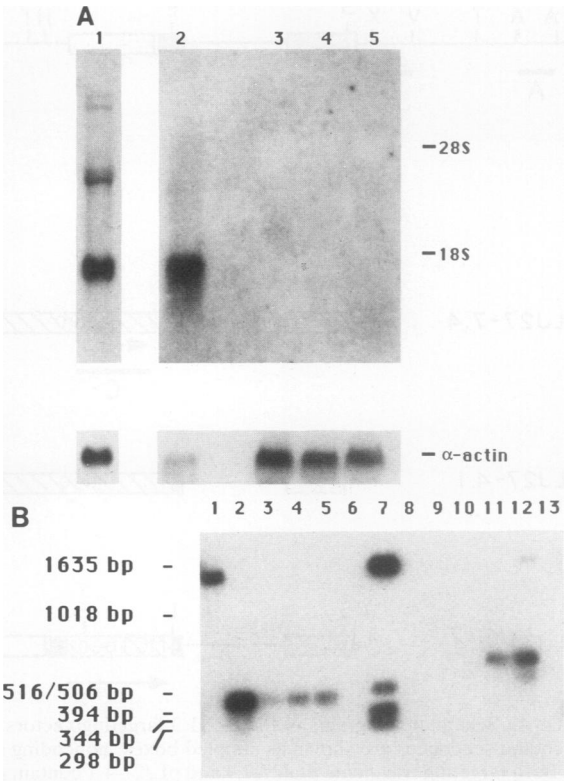

FIG. 2. Expression of the Hox1.3 gene in the CCE-ES cell line. (A) Northern blot analysis of RNA isolated from parental and targeted ES cell lines. The RNAs were fractionated by electrophoresis through a formaldehyde-agarose gel, transferred to Hybond membrane, and hybridized to a ^{32}P-labeled probe contained in the first exon of Hox1.3 gene (see Materials and Methods). To demonstrate RNA integrity, the blots were rehybridized to a mouse α-actin probe. Lanes: 1, 3 μg of poly(A)$^+$ RNA from 12.5-day-old embryos; 2, 5 μg of poly(A)$^+$ RNA from STOneo^r cell line; 3, 4, and 5, 20 μg of poly(A)$^+$ from parental and two different pLJ27-targeted ES cell lines. (B) PCR analysis of RNA isolated from parental and targeted ES cell lines. PCR products (see Materials and Methods) were fractionated by electrophoresis through a 1.5% agarose gel, transferred to a GeneScreen Plus membrane, and hybridized to a ^{32}P-labeled probe contained in the first exon of the Hox1.3 gene. For the samples in lanes 1 to 6, Hox1.3-specific primers (Fig. 1) were used for PCR amplification, while for the samples in lanes 8 to 13, one Hox1.3 (from exon 1)- and one *neo*-specific primer were used for PCR amplification. Lanes: 1 and 8, CCE-ES cell DNA; 2 and 9, STOneo^r cell RNA; 3 and 10, CCE-ES cell RNA; 4, 5, 11, and 12, ES cell RNA from two different pLJ27-targeted clones; 6 and 13, no nucleic acid.

RESULTS

Expression of Hox1.3 gene in ES cells. Depending on the transcriptional activity of the target locus, different strategies can be used to introduce and select for homologous recombinant events. To determine the level of expression of the Hox1.3 gene, we first performed Northern blot analysis with poly(A)$^+$ RNA extracted from the CCE-ES cell line. Importantly, the CCE cells were cultured for two passages on gelatin-coated plates to avoid feeder cell contamination. As shown in Fig. 2A, no Hox1.3 transcript could be detected

in the ES cell RNA after 3 days of autoradiographic exposure or after 28 days (not shown), while a strong signal was seen for the 2-kb Hox1.3 transcript in the STOneo^r sample. As a control, the probe was also hybridized to poly(A)$^+$ RNA isolated from 12.5-day-old embryos, which revealed the predicted Hox1.3-specific transcription pattern (29). In order to ascertain the sensitivity of our Northern blot, the same blot was hybridized with a mouse dihydrofolate reductase cDNA probe (41). After a 4-day exposure, we detected the two expected dihydrofolate reductase transcripts (1.6 kb and 750 bases [38]), which compose less than 0.1% of poly(A)$^+$ RNA in mouse cells (2; data not shown). Moreover, using the more sensitive RNase protection assay, we failed to detect Hox1.3-hybridizing sequences in ES cell RNA (data not shown).

In order to determine whether Hox1.3 gene transcription is below the level of detection of standard procedures, we used the PCR to amplify Hox1.3 cDNA-specific fragments (45). By using primers derived from the two Hox1.3 gene exons that allowed discrimination of contaminating genomic DNA sequences from transcript-derived Hox1.3 cDNA, it was possible to amplify a 453-bp fragment specific to the Hox1.3 mRNA in the ES cell RNA samples (Fig. 2B, lanes 3, 4, and 5). The inability to detect Hox1.3 transcripts in ES cells by Northern blotting or RNase protection and the ability to detect them by PCR indicate the extremely low levels of Hox1.3 mRNA in these cells.

Targeting vector design. The successful application of the promoterless gene-targeting approach has, to date, been confined to genes that are highly expressed (7, 17, 36, 37, 40). In order to test whether this procedure can be used to target genes expressed at low levels such as Hox1.3, we designed a replacement vector in which a promoterless bacterial *neo* gene lacking an AUG translational initiation codon was inserted into Hox1.3 genomic sequences (pLJ27 [Fig. 1]). The targeting fragment pLJ27-7.4 contains Hox1.3 sequences from positions −129 bp to +7.3 kb (relative to the transcription initiation site). It contains a putative TATA box as well as two Hox1.3 binding sites (29, 46). However, upstream sequences up to −235 bp are not sufficient to direct transcription of the Hox1.3 gene in transgenic mice (46). Therefore, we anticipated that random integration of this targeting vector should not lead to expression of the Hox1.3-*neo* fusion gene in a position-independent manner. A second targeting fragment, pLJ27-4.1, does not contain any putative promoter sequences and possesses less homology with the Hox1.3 gene (from positions +74 bp to +4.1 kb). Thus, the *neo* gene contained in the pLJ27-7.4 and pLJ27-4.1 targeting fragments can be expressed only as a fusion protein under the transcriptional control of an endogenous promoter. A homologous recombinant event, mediated by a double reciprocal crossover in the Hox1.3-flanking DNA, would position *neo* in frame after amino acid 214 in the homeodomain of the Hox1.3 gene. The translation of the hybrid Hox1.3-*neo* mRNA would produce a fusion protein of 477 amino acids capable of conferring G418 resistance but destroying the putative DNA binding homeodomain (46).

As it was not possible to predict how effective the promoterless vector approach would enrich for targeting events at the Hox1.3 locus, we constructed a PNS replacement vector in parallel (pLJ31 [Fig. 1]). An MC1neoA$^+$ cassette was inserted into the homeodomain of the second exon of the Hox1.3 gene. In addition, a HSV-*tk* counterselection cassette was included at the 3′ extremity of the Hox1.3 homology. Random integration would result in retention of the entire vector sequence resulting in expression of the HSV-*tk* gene thus conferring GANC sensitivity to the cells (25). Integration by homologous recombination of the vector with the target gene will result in the loss of the HSV-*tk* sequences, allowing the cells to survive GANC administration. The insertion of the MC1neoA$^+$ cassette into the homeodomain will prevent the production of a full-length Hox1.3 protein.

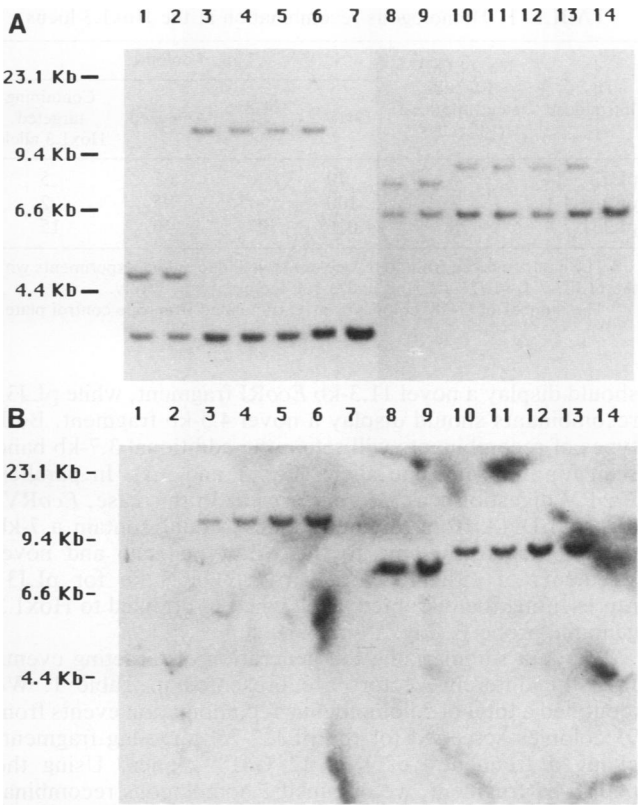

FIG. 3. Southern blot analysis of genomic DNA from individual homologous recombinant cell lines. DNA isolated from parental CCE-ES cells and six targeted clones was digested with *Eco*RI (lanes 1 to 7) or with *Eco*RV (lanes 8 to 14) and analyzed by Southern blotting. Hybridization was either with the 5′ *Acc*I-*Acc*I Hox1.3 genomic probe A for the *Eco*RI digestion or with the 5′ *Eco*RV-*Xba*I genomic Hox1.3 probe B for the *Eco*RV digestion (A) and with the *neo* probe (B). Lanes: 1, 2, 8, and 9, DNA from pLJ31-targeted clones; 3, 4, 10, and 11, DNA from pLJ27-7.4-targeted clones; 5, 6, 12, and 13, DNA from pLJ27-4.1-targeted clones; 7 and 14, CCE-ES cell DNA.

Homologous recombination at the Hox1.3 locus. All experiments were carried out by using the CCE cell line, which has been shown to reproducibly colonize the male germ line of chimeras with high efficiency (9, 33, 35). The different targeting fragments pLJ27-7.4, pLJ27-4.1, and pLJ31-7.4 were introduced into ES cells by electroporation, and the cells were submitted to selection with the appropriate drugs (see Materials and Methods). After selection, drug-resistant colonies were clonally expanded and genomic DNA was isolated from cells grown for two passages without feeder cells to avoid contamination by STO cell DNA.

DNAs were initially analyzed by Southern blotting for diagnostic Hox1.3 fragments. Homologous recombination of either construct into the Hox1.3 locus should destroy the *Eco*RI site contained in the homeobox (Fig. 1). Thus, when Hox1.3 genomic probe A is used, pLJ27 recombinants

TABLE 1. Homologous recombination at the Hox1.3 locus

DNA introduced	No. of CCE-ES cells electroporated (10^7 cells)[a]	No. of colonies:			
		G418^r	G418^r GANC^r	Analyzed	Containing targeted Hox1.3 allele
pLJ27-7.4	8	70		61	5
pLJ27-4.1	8	100		95	2
pLJ31-7.4	28	4,016[b]	307	140	15

[a] The data are taken from four, one, and two independent experiments with the pLJ27-7.4, pLJ27-4.1, and pLJ31-7.4 vectors, respectively.
[b] The number of G418^r colonies was extrapolated from one control plate.

should display a novel 11.3-kb *Eco*RI fragment, while pLJ31 recombinants should display a novel 4.5-kb fragment. Both types of recombinants will retain the additional 3.7-kb band seen for the wild-type allele (Fig. 1 and 3A). In parallel, *Eco*RV digestion was also performed. In this case, *Eco*RV-digested DNA from targeted clones should contain a 7-kb fragment corresponding to the wild-type gene and novel fragments of either 9 kb for pLJ27 or 8 kb for pLJ31 representing the disrupted allele when hybridized to Hox1.3 genomic probe B (Fig. 1 and 3A).

The data summarizing the generation of targeting events with the different vectors are presented in Table 1. We identified a total of 5 homologous recombination events from 93 colonies screened for the pLJ27-7.4 targeting fragment, giving a frequency of 1 in 12 G418^r clones. Using the pLJ27-4.1 fragment, we obtained 2 homologous recombination events from 95 colonies screened, giving a frequency of 1 in 48 G418^r clones. In the case of the pLJ31 vector, 15 homologous recombinants were obtained of 140 doubly resistant clones screened, which gives a frequency of 1 in 9 G418^r GANC^r clones.

For all these recombinant clones, hybridization of the blots with a probe containing *neo* sequences demonstrated the presence of a single band corresponding to the disrupted Hox1.3 allele (Fig. 3B), which indicates that only a single integration event occurred. Surprisingly, we observed that for a number of clones the intensity of hybridization to the wild-type allele exceeded that for the targeted allele (Fig. 3A, lanes 3 to 6 and 10 to 13). We therefore performed a number of experiments to confirm the homologous recombination events and to analyze the fine structure of the targeted locus. First, we hybridized representative blots with a pUC18 probe to eliminate the possibility that recombination occurred by a single crossover insertion event at the Hox1.3 locus to generate a partial duplication. No pUC sequences were detected in any of the pLJ27 and pLJ31 recombinant clones, indicating that a double crossover replacement event occurred (data not shown). Second, we verified that the 5′ and 3′ sequences adjacent to the integration site corresponded to that predicted for a homologous recombination event at the locus. Since we observed *Eco*RI fragments of the predicted size in the recombinant clones (Fig. 1 and 3A), we digested a representative pLJ27 recombinant with enzymes that cleave outside the targeting vector but within the *Eco*RI fragment. Such digests would enable us to detect rearrangements in the 5′ and 3′ sequences flanking the Hox1.3 integration sites. DNA was digested with *Stu*I, *Xba*I, and *Eco*RV and hybridized with the 5′ external probe A (Fig. 1), to enable us to detect rearrangements within 3, 15, and 5 kb of the integration site, respectively. Similarly, a parallel blot was hybridized with a 3′-specific probe (probe D, Fig. 1), which would enable us to detect rearrangements within about 2 kb on the 3′ side of the integration site. As shown in Fig. 4 (right panel), hybridization with the 5′ probe yielded identically sized fragments for all three digests of either wild-type or targeted cell line DNA, indicating that no rearrangements had occurred in the 15-kb sequences 5′ of the integration site. A similar analysis with a 3′ probe indicates that for each enzyme, identically sized fragments are present in both the wild-type and targeted cell DNA (Fig.

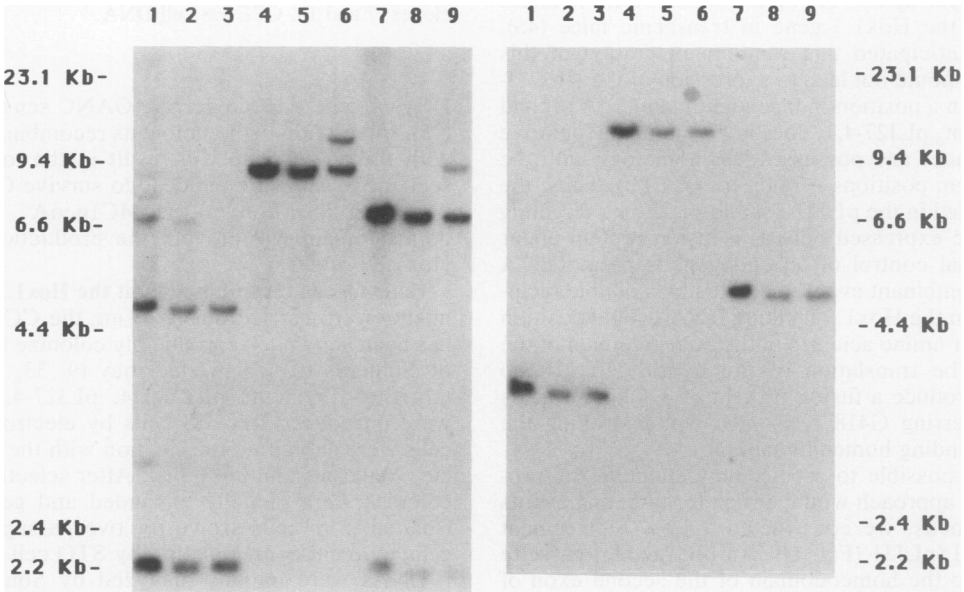

FIG. 4. Southern blot analysis of genomic DNA isolated from STOneo^r cells, parental CCE-ES cells, and a targeted clone pLJ27-7.4, digested with *Stu*I (lanes 1 to 3), *Xba*I (lanes 4 to 6), and *Eco*RV (lanes 7 to 9). Hybridization was either with the 3′ *Hin*dIII-*Hin*dIII Hox1.3 genomic probe D (left panel) or with the 5′ *Acc*I-*Acc*I Hox1.3 genomic probe A (right panel). Lanes: 1, 4, and 7, STOneo^r DNA; 2, 5, and 8, CCE-ES cell DNA; 3, 6, and 9, DNA from targeted clone pLJ27-7.4.

4, left panel). The additional higher-molecular-weight band in lanes 6 and 9 represents the targeted allele. Thus, we obtained no indication that a major rearrangement of either vector or host DNA had occurred during the homologous recombination events that generated the disrupted allele.

We found that pLJ27 homologous recombinants produce Hox1.3-*neo* fusion transcripts in addition to normal Hox1.3 transcripts. Similar to the results obtained for the Hox1.3 expression in ES cells, we did not detect Hox1.3-*neo* transcripts in the homologous recombinant clones by Northern analysis (data not shown). However, by using primers derived from the first exon of Hox1.3 gene and the *neo* sequences, it was possible to amplify a 645-bp fragment specific to the Hox1.3-*neo* transcript in the ES recombinant samples (Fig. 2B, lanes 11 and 12). This fragment also hybridizes to a *neo* probe (data not shown). Thus, as expected, *neo* expression is directed by the endogenous Hox1.3 promoter and a functional *neo* fusion protein confers resistance to G418.

DISCUSSION

Gene disruption in ES cells is a powerful method to study and define the role of specific gene products in vivo. Much effort has been made to define the parameters which influence the ability of introduced DNA sequences to undergo homologous recombination with genomic sequences. For example, localized chromatin structure, the presence of repetitive elements, and the transcriptional state could all potentially affect recombination at a given chromosomal site. However, to date, no consensus that allows an accurate prediction of the rate of homologous recombination at a particular locus exists. Given that detection of homologous recombination is obscured by a high background of random integration events, a number of experimental strategies have been devised to selectively eliminate cells carrying randomly integrated copies of vector sequences.

The use of promoterless targeting vectors, incorporating the *neo* selection gene to recover rare homologous recombination events, has previously been shown to allow the efficient disruption of genes that are highly expressed in ES cells (7, 36, 40). In this report, we have extended these studies by demonstrating the usefulness of this approach for introduction of mutations in genes with barely detectable transcription rates in ES cells such as Hox1.3. A targeting vector incorporating 7.4 kb of genomic homology to Hox1.3 gave a ratio of 1 homologous to 12 random recombination events. Assuming that a 100-fold enrichment is obtained by the exclusion of promoter elements, the targeting frequency for this locus is on the order of 1 in 1,200 integration events. This compares extremely favorably with previous data obtained at other loci (7, 17, 36, 37, 40) and, importantly, suggests that transcriptional activity of the target gene does not necessarily correlate with the ability for homologous recombination to occur at this locus. Moreover, analysis of RNA levels in cell lines carrying a disrupted Hox1.3 allele clearly showed that the G418 resistance phenotype can be conferred by extremely low levels of the Hox1.3-*neo* fusion transcript. In this case, the predicted protein, containing a NH_2-terminal region composed of 214 amino acids derived from the normal Hox1.3 protein and a COOH terminus from the *neo* protein, functions efficiently. Thus, these data are in agreement with other experimental evidence showing that *neo* in the context of a large fusion protein can retain its biological activity (36).

Previous studies, modeled at the HPRT locus, have shown a relationship between the rate of homologous recombination and the length of homology present in the targeting vector (42). The same relationship was observed by using replacement vectors at the autosomally encoded Hox1.3 locus. Reduction of the length of homology included on the 3′ side of the *neo* gene by some 3.3 kb, reduced the rate of homologous recombination by a factor of four.

An alternative strategy used to minimize the recovery of illegitimate recombination events is the addition of a second counterselection cassette flanking the genomic homology in the targeting vector (25). This strategy has been used successfully to target a number of loci that are apparently transcriptionally inactive in ES cells (18, 27, 43). We compared the efficiency of homologous recombination at the Hox1.3 locus by using either a promoterless or a PNS vector containing the identical genomic homology. The enrichment of 13-fold, obtained after counterselection with GANC, was comparable to that reported by others (9, 27, 30), and approximately one of nine doubly resistant clones proved to contain homologous recombinants at the Hox1.3 locus. Thus, overall, the efficiency of recovering targeted clones is similar for both vector designs.

Surprisingly, we observed during the Southern blot analysis that, regardless of the targeting vector employed, there were a number of clones in which hybridization to fragments derived from the wild-type allele was twofold more intense than that observed for the mutated allele. The result may suggest that these clones contain three Hox1.3 alleles, one of which represents the mutated allele. We can envision several mechanisms by which the apparent duplication can be generated. For example, partial duplication could be produced by a single crossover event between the targeting vector and the Hox1.3 locus. Such a model is ruled out, since no plasmid sequences were detected in DNA samples from the recombinants. In addition, it could be argued that the targeting vector integrates into random sites that fortuitously generate *Eco*RI and *Eco*RV fragments identical in size to that predicted for a correctly targeted event. However, none of the DNA analyses performed provided evidence for random integration; diagnostic Hox1.3 restriction fragments covering some 15 kb of 5′ flanking sequences and 2 kb of 3′ flanking sequences were generated by using a number of restriction enzymes. A final possibility is that during the recombination process, endogenous Hox1.3 sequences were added to the targeting vector. Subsequently, the altered vector could integrate at the Hox1.3 locus to form a tandem duplication or integrate at a new genomic site (1). This latter explanation can be addressed by fluorescence in situ hybridization to metaphase chromosomes to confirm the presence of only two Hox1.3 alleles.

While ES cells are developmentally equivalent to normal inner cell mass cells of the peri-implantation embryo (5), their pattern of gene expression in tissue culture differs both quantitatively and qualitatively from that displayed by early embryo cell populations (22; unpublished data). One example of this discrepancy is illustrated by the Hox1.3 gene, which is normally activated at day 8 of development when its expression is highly restricted (11). The very low level of expression in ES cells that was detected by PCR analysis was sufficient to allow us to use a promoterless *neo* targeting strategy to recover clones of cells carrying a mutation in an endogenous allele with very high efficiencies. This approach should be equally applicable to other genes that are normally only expressed at later stages of embryogenesis, but which are transcriptionally active in ES cells in culture.

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