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TECHNICAL REPORT

Rapid confirmation of gene targeting in embryonic stem cells using two long-range PCR techniques

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Gene targeting in mouse embryonic stem (ES) cells generally includes the analysis of numerous colonies to identify a few with mutations resulting from homologous recombination with a targeting vector. Thus, simple and efficient screening methods are needed to identify targeted clones. Optimal screening approaches require probes from outside of the region included in the targeting vector to avoid detection of the more common random insertions. However, the use of large genomic fragments in targeting vectors can limit the availability of cloned DNA, thus necessitating a strategy to obtain unique flanking sequences. We describe a rapid method to identify sequences adjacent to cloned DNA using long-range polymerase chain reaction (PCR) amplification from a genomic DNA library, followed by direct nucleotide sequencing of the amplified fragment. We have used this technique in two independent gene targeting experiments to obtain genomic DNA sequences flanking the mouse cholecystokinin (CCK) and gastrin genes. The sequences were then used to design primers to characterize ES cell lines with CCK or gastrin targeted gene mutations, employing a second long-range PCR approach. Our results show that these two long-range PCR methods are generally useful to rapidly and accurately characterize allele structures in ES cells.

Keywords: polymerase chain reaction; stem cells; gene targeting; gastrin; cholecystokinin

Introduction

It is becoming common to engineer specific gene mutations in the mouse germline by gene targeting in embryonic stem (ES) cells. This is accomplished by using a targeting vector designed to replace the corresponding endogenous gene by homologous recombination. Since it is much more common for the targeting vector to insert into a random chromosomal site than a homologous one, it is necessary to screen colonies by Southern hybridization or polymerase chain reaction (PCR) to identify rare targeted clones. Several improvements have been described that increase the efficiency of recovery of homologous recombinants, including positive/negative selection schemes designed to kill clones containing random insertions of the targeting vector (Mansour *et al*., 1988; Yagi *et al*., 1990), as well as the use of isogenic DNA in the targeting vector to increase the rate of homologous recombination (te Riele *et al*., 1992). However, in spite of these improvements, targeting efficiencies typically range from 0.5% to 10% of ES clones that survive selection. Thus, it is often necessary to screen hundreds of ES cell clones in order to identify sufficient numbers of correctly targeted cell lines to transfer the mutation to the mouse germline. Methods that simplify the screening or analysis of putative targeted clones are needed to facilitate this process.

The use of long genomic regions (5–10 kb) in targeting vectors has also been shown to increase the frequency of homologous recombination in cultured cells (Thomas and Capecchi, 1987; Shulman *et al*., 1990; Hasty *et al*., 1991). However, the inclusion of long

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genomic regions can sometimes limit the amount of remaining cloned material to develop flanking region probes. Optimal screening approaches for identification of targeted clones require unique sequences flanking the targeting vector for use as a hybridization probe or PCR primer. Using flanking sequences avoids detecting the more common random integrants during the screening process. In some cases, the identification of unique flanking sequences requires the characterization of additional genomic DNA, either because the extent of previously cloned DNA was used to construct the targeting vector, or because of lengthy repetitive sequences. Thus methods that rapidly characterize sequences adjacent to cloned DNA would facilitate gene targeting experiments.

In this report we describe a rapid and accurate method, using two different long-range PCR techniques, to obtain flanking sequences and characterize ES clones. Long-range library PCR is first used to obtain new genomic DNA sequence flanking a region of interest. The new DNA sequence is then used to design a primer for analysis of allele structures in ES cells using a second long-range PCR assay. This approach has been used to characterize putative targeted ES clones from two independent experiments to demonstrate its general usefulness.

Materials and methods

Long-range library PCR

Long-range PCR was performed on a mouse genomic DNA library with one primer specific for the CCK gene (GS-C) or the gastrin gene (GS-G) and a second primer corresponding to vector sequence (LT3 or LT7), using the Expand Long Template PCR System (Boehringer-Mannheim). The primer sequences were: 5′CGCTCATCCTGTTTAGTGGAGGTCAAGAAG (GS-C), 5′GTTTCTGGATTTTTGGTCTGTTTCTGGAG (GS-G), 5′GCGAGCTCAATTAACCCTCACTAAAGGG (LT3) and 5′GCGAGCTCTAATACGACTCACTATAGGG (LT7). PCR reactions (50 μl) included 1 μl of a mouse 129/SvJ genomic library in the Lambda FIX II vector (Stratagene), 350 μM dNTPs, 1 μM of each primer and 2.6 units Expand enzyme mix in Expand buffer 1 (50 mM Tris-HCl, pH 9.2, 16 mM $(NH_4)_2SO_4$, 1.75 mM $MgCl_2$). Amplification was done in 0.5 ml reaction tubes using an MJ Research thermocycler with an initial denaturation step of 94 °C for 5 min followed by 10 cycles of 94 °C for 30 s, 60 °C for 30 s, 68 °C for 10 min, and then 20 cycles of 94 °C for 30 s, 60 °C for 30 s, 68 °C for 15 min, with a final extension step of 68 °C for 7 min.

Aliquots (10 μl) of long range library PCR reactions were electrophoresed on 0.7% agarose gels, transferred to Zetaprobe membrane (BioRad) and hybridized with a CCK-region or gastrin-region probe as previously described (Samuelson *et al*., 1995). The 0.2 kb gastrin-region probe was PCR amplified from λgas1 DNA (Friis-Hansen *et al*., 1996), using primer pair LT7/GS-G. The 0.2 kb CCK-region probe was PCR-amplified from a mouse CCK plasmid clone (Vitale *et al*., 1991), using the GS-C primer paired with a vector specific primer. Probes were excised from low melt agarose, purified using the Qiaex gel extraction kit (Qiagen) and ^{32}P-labelled by random priming (Feinberg and Vogelstein, 1983). After hybridization to the long-range library PCR products, one gastrin hybridizing fragment of 2.7 kb, and two CCK hybridizing fragments of 1.2 and 2.1 kb were excised and purified using the Qiaex gel extraction kit (Qiagen). The purified DNAs were sequenced by the University of Michigan Sequencing Core on an ABI 373 DNA sequencer using primer GS-C or GS-G.

Analysis of allele structures in mouse ES cells by long-range PCR

Mouse R1 cells (Nagy *et al*., 1993) were cultured on neo-resistant mouse embryonic fibroblast feeders as described (Kendall *et al*., 1995), with the addition of 10^3 units of recombinant leukaemia inhibitor factor (ESGRO; Gibco BRL) per ml of culture medium. The CCK and gastrin targeting experiments will be described in detail elsewhere (Lay *et al*., in preparation; Friis-Hansen *et al*., 1988). In brief, after electroporation of a targeting vector, colonies were selected in G418 (300 μg/ml) (CCK), or G418 and gancyclovir (2 μM) (gastrin), and isolated in individual wells of a 96-well culture dish. Genomic DNA was prepared from replicate confluent 96-well dishes (Ramirez-Solis *et al*., 1992) and colonies were screened for CCK or gastrin gene targeting using conventional PCR. Allele structures in putative targeted clones were then characterized by long-range PCR using 50–200 ng of ES cell genomic DNA and the long range amplification conditions described above. The primer sequences were: 5′AGTCACTGTGCTCTGCCTACTCG-TGGCACA (C1), 5′TAGGACTGCCATCACCACGCACAGACATAC (C2), 5′AAACCAGGCAAAGCGCCATTCGCCATTCAG (Lac), 5′CTGTATGATCAGCTATCCTG- CAACATTCCC (G1), 5′CCAAAGTCCATCCATCCGTA- GGCCTCTTCT (G2), and 5′AAGAACTCGTCAAGAAGGCGATAGAAGGCG (Neo). Genomic Southern hybridization analysis was performed on *Hin*d III digested ES cell DNAs using a 0.8 kb *Eco* RI/*Bgl* II CCK-region probe (Fig. 2A) as described previously (Samuelson *et al*., 1995).

Results and discussion

Long-range library PCR

The long-range library PCR method is used to amplify genomic DNA immediately adjacent to a cloned region as

an alternative to screening a genomic DNA library. Our goal was to obtain DNA sequence upstream of the region encompassed by a targeting vector to facilitate the characterization of mouse ES cell colonies. To obtain genomic DNA sequence upstream of the cloned region, a gene-specific primer (GS) from the 5′ end of the known sequence was paired with a vector-specific primer (LT3 or LT7) to amplify fragments from a mouse genomic library (Fig. 1). Long-range PCR was used, since this technique easily allows the amplification of fragments in the size range of lambda library inserts (Barnes, 1994; Cheng *et al*., 1994). After amplification, gene specific products were identified by Southern hybridization analysis, and selected fragments were gel purified and subjected to automated nucleotide sequencing with the gene specific primer.

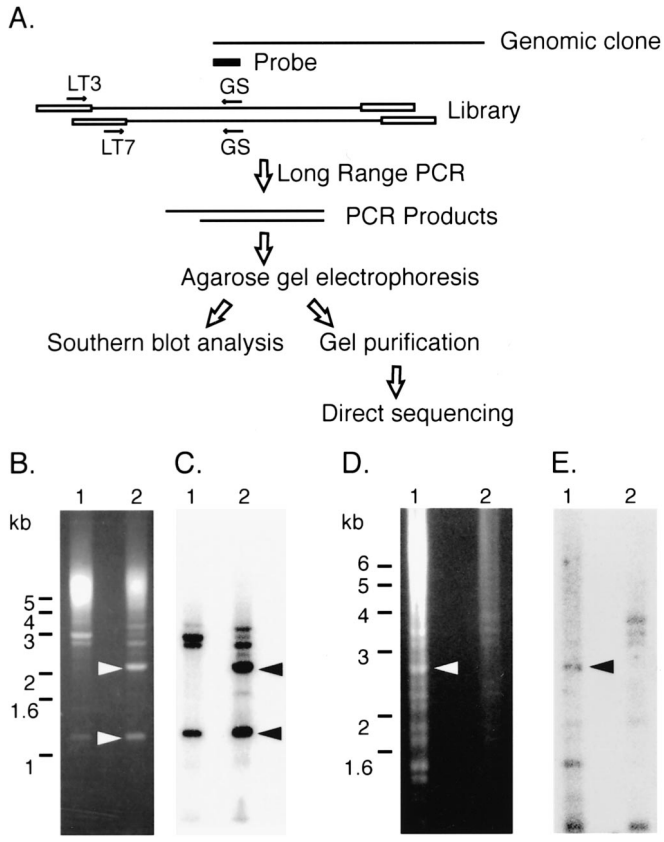

Fig. 1. Long-range library PCR amplification of the regions flanking the mouse gastrin and CCK genes. (A) Schematic diagram of the long-range library PCR procedure. The positions of the PCR primers (arrows) and hybridization probe are diagrammed. Lines, genomic sequence; open boxes, lambda vector arms. (B–E) Analysis of PCR amplification products from upstream of CCK (B, C; primer GS-C) and gastrin (D, E; primer GS-G) by agarose gel electrophoresis (B, D) and Southern blot (C, E). The products were generated with either the LT3 (lanes 1) or LT7 (lanes 2) primer paired with a gene specific primer. Arrowheads indicate the fragments that were purified and sequenced.

We used the long-range library PCR method for manipulation of the mouse cholecystokinin (CCK) and gastrin genes, which are unlinked single copy genes (Vitale *et al*., 1991; Friis-Hansen *et al*., 1996). The CCK gene specific primer (GS-C) was located 5 kb upstream of the CCK gene, and the gastrin primer (GS-G) was located 8 kb upstream of the gastrin gene. Specific amplification products were observed when the GS-C or GS-G primers were paired with the vector specific primers LT3 and LT7 (Fig. 1B and 1D). Southern hybridization analysis with CCK or gastrin region probes confirmed that the predominant amplification products were gene specific (Fig. 1C and 1E). Two CCK region fragments of 1.2 and 2.1 kb amplified with the GS-C/LT7 primers (Fig. 1B, arrowheads), and one gastrin region fragment of 2.7 kb amplified with the GS-G/LT3 primers (Fig. 1D) were gel purified and subjected to automated nucleotide sequencing with the corresponding GS-C or GS-G primer. The sequence data confirmed that the fragments contained the desired upstream sequences, since the initial sequence was identical to the 5′ end of the CCK or gastrin clone, extending further in the upstream direction (not shown).

In contrast to the long-range library PCR method, other methods that amplify unknown flanking DNA fragments often include difficult or time-consuming steps such as randomly primed PCR or adapter ligations (Ochman *et al*., 1988; Rosenthal and Jones, 1990; Lagerstrom *et al*., 1991; Parker *et al*., 1991; Mizobuchi and Frohman, 1993; Siebert *et al*., 1995). Advantages of the long-range library PCR technique include its simplicity and the speed with which new genomic DNA sequence can be obtained. Our technique takes advantage of the numerous lambda phage genomic DNA libraries that are commercially available. Once the gene-specific primer is synthesized, one can obtain additional sequence either upstream or downstream of a known region in a few days, as demonstrated by our results with the mouse CCK and gastrin genes. In addition to direct DNA sequencing, specific amplification products from the long-range library PCR can be cloned for the development of hybridization probes. The characterization of flanking region DNA has a variety of applications, including the design of new primers and probes for analysis of gene targeting in mouse ES cells.

Long-range PCR analysis of gene targeting in ES cells

We used the upstream region sequences obtained by long-range library PCR to characterize CCK and gastrin allele structures in gene targeting experiments. The CCK and gastrin gene targeting vectors included long (6–8 kb) homology segments at their 5′ ends, short (< 1.5 kb) segments at their 3′ ends, and neomycin phosphotransferase (neo) selection cassettes (Figs 2A and 3A). Potentially targeted ES cell clones were first identified by conven-

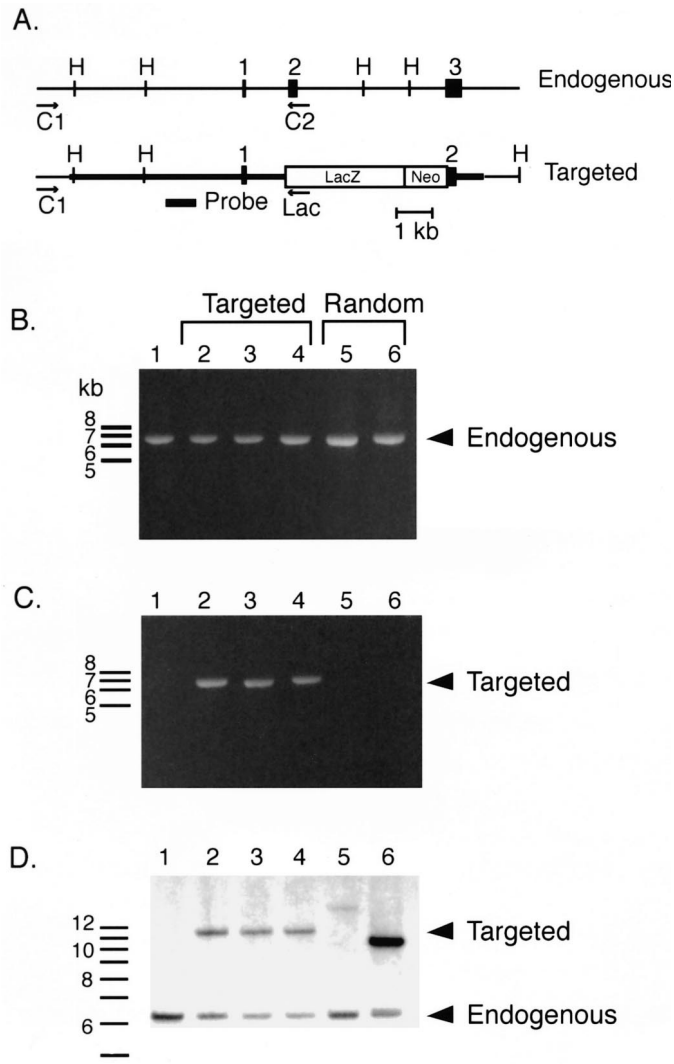

Fig. 2. Characterization of CCK allele structures in ES cell clones by long-range PCR and Southern blot. Five representative ES cell colonies are shown (lanes 2–6) along with the unmanipulated ES cell line (lane 1). (A) Schematic diagram showing the endogenous and targeted alleles. The CCK targeting vector deleted 168 bp of exon 2, substituting in its place a *lacZ* reporter gene (from plasmid pCH110; Pharmacia), and a neo selection cassette controlled by the phosphoglycerate kinase-1 promoter (Tybulewicz *et al*., 1991). The thick line on the targeted allele represents the genomic regions included in the targeting vector. H, *Hin*d III. (B) Long-range PCR products generated using the primer pair C1/C2. The 6.5 kb amplified fragment is specific for the endogenous gene structure. (C) Long-range PCR products generated using the primer pair C1/Lac. The 6.5 kb amplified fragment is specific for the targeted gene structure. (D) Southern blot analysis of ES cell clones. Samples were digested with *Hin*d III; probe is shown above in (A).

tional screening approaches. ES clones were cultured in 96-well plates, genomic DNA was isolated from replica plates and screened by PCR amplification of a fragment encompassing the short homology segment (not shown). Southern blot analysis is generally performed as a secondary screen to confirm that the targeting vector was correctly inserted by homologous recombination. However, this analysis can be quite slow since the Southern procedure extends over several days. Furthermore, since the 96-well DNA extraction produces limited amounts of DNA, PCR strategies that use less DNA than Southern blots allow multiple assays to be performed. Thus we designed a rapid long-range PCR assay to characterize putative targeted clones, taking further advantage of the 96-well DNAs. Since the initial screening consisted of analysis at the 3′ end, the secondary screening focused on the 5′ recombination region. It is important to evaluate both ends of the locus to confirm the double recombination needed to achieve gene replacement (Hasty and Bradley, 1993).

A total of 16 ES cell clones containing potentially targeted CCK mutations were analysed. Long-range PCR reactions specific for the endogenous and targeted gene structures were performed using the upstream primer (C1), designed from the new genomic sequence obtained by long-range library PCR with allele specific downstream primers (Fig. 2). The endogenous allele was amplified with primer C2, which corresponds to gene sequences deleted from the targeted allele. As expected, the 6.5 kb endogenous gene fragment was amplified from all ES clones analysed with the C1/C2 primer pair (Fig. 2B). The targeted CCK gene structure was identified by pairing the C1 primer with a *lacZ*-region primer (Lac) to amplify a 6.5 kb fragment. The C1/Lac primers amplified the correct targeted fragment from ten clones (Fig. 2C, lanes 2–4); no product was obtained from unmanipulated ES cells (lane 1) or the 6 clones containing incorrect insertions of the targeting vector (lanes 5 and 6). Genomic Southern analysis was performed to confirm the allele structures identified by PCR typing. ES cell DNAs were digested with *Hin*d III and probed with a CCK probe (Fig. 2D) that detects both targeted and random integrations of the targeting vector. An 11.1 kb band specific for the targeted gene structure was observed in the ten lines identified as correctly targeted by long-range PCR. Since this probe is internal to the targeting vector, the random integrants (Fig. 2D lanes 5 and 6) have bands which differ in size from the 11.1 kb targeted band. Also in agreement with the long-range PCR results, all 16 of the ES cell lines contained a 6.4 kb band corresponding to the endogenous allele. Additional Southern analysis was performed to examine the allele structures in greater detail (data not shown). The long-range PCR results were in complete concordance with the results from the Southern analysis.

A similar long-range PCR strategy was used to analyse ES cell lines from a gastrin gene targeting experiment (Fig. 3). In this experiment the targeting vector deleted a 3.1 kb region containing the complete gastrin gene, and

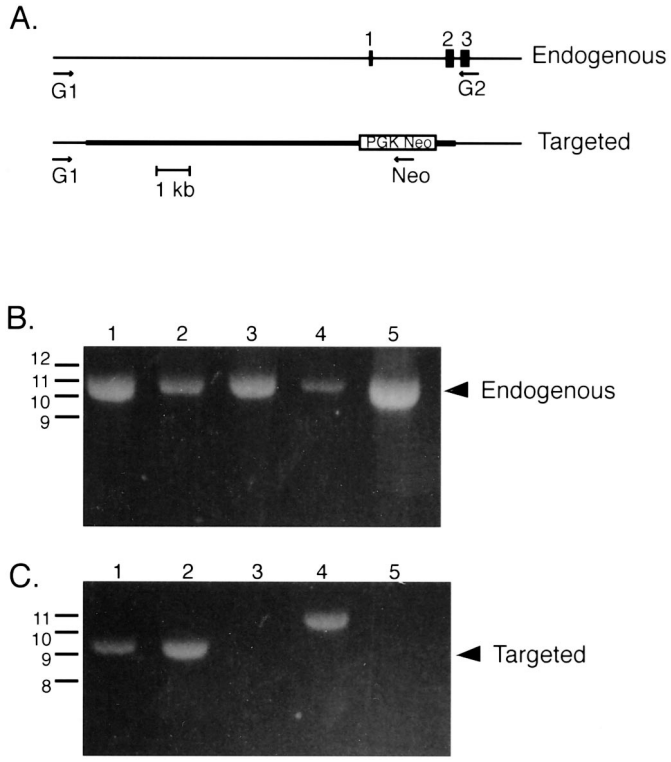

Fig. 3. Characterization of gastrin allele structures in ES cell clones by long-range PCR. Four independent ES cell colonies are shown (lanes 1–4) along with the original ES cell line (lane 5). (A) Schematic diagram of the endogenous and targeted alleles. The gastrin gene has been completely deleted in the targeted allele and substituted by a *neo* selectable cassette controlled by the phosphoglycerate kinase-1 promoter (Tybulewicz *et al*., 1991). The thick line on the targeted allele represents the genomic regions included in the targeting vector. (B) Long-range PCR products generated using the primer pair G1/G2. The 11 kb amplified fragment is specific for the endogenous gene structure. (C) Long-range PCR products generated using the primer pair G1/Neo. The 9.3 kb amplified fragment is specific for the targeted gene structure.

replaced it with a *neo* selection cassette. A gastrin upstream primer (G1) designed from the flanking DNA sequence obtained by long-range library PCR was paired with primers specific for the endogenous gene (G2) or the targeted gene (Neo) to amplify the two alleles from putative targeted clones. An 11 kb fragment specific to the endogenous gene was amplified from 11 potentially targeted clones with the G1/G2 primers (Fig. 3B). The G1/Neo primer pair specific to the targeted gene amplified a 9.3 kb fragment from 8 of the 11 clones, thus identifying them as correctly targeted (Fig. 3C). Those clones that did not amplify the 9.3 kb fragment included two with random inserts (lane 3) and one with an incorrect gene targeting event (lane 4). Southern hybridization analysis performed on the correctly targeted clones (data not shown), demonstrated the accuracy of the long-range PCR analysis.

As shown in two independent targeting experiments, the long range PCR techniques described here provide a rapid and accurate method to characterize allele structures in ES cell clones. The long-range library PCR technique was first used to design specific oligonucleotide primers upstream of the region contained in the targeting vectors. The upstream primers were then used to characterize gene structures in ES cells with a second long-range PCR assay. Any PCR approach requires much less genomic DNA (approximately 100 ng) than a Southern blot (often 10 μg are used). Long-range PCR analysis of ES cell clones can be utilized, as we demonstrate here, as a rapid confirmation of allele structures in gene targeting experiments. This allows accurately targeted clones to be quickly identified. Rapid identification may render unnecessary the common practice of freezing ES clones during screening (Ramirez-Solis *et al*., 1992), and thus significantly shorten the time required to transfer the targeted mutation to the mouse germline. ES clones can be passaged in 96-well plates during the PCR screening, subsequently expanding only positive ES clones for further analysis and microinjection into blastocysts. Although the PCR analysis accurately identified correctly targeted clones in our study, it would be prudent to also perform at least one Southern blot before microinjection since PCR would miss the identification of clones that have inserted multiple copies of the targeting vector.

Long-range PCR could also be used for the primary screen of ES colonies, allowing PCR analysis in cases where both of the targeting vector arms are too large to permit conventional PCR screening. Previously, the use of PCR as a primary screening strategy limited the size of one arm of the targeting vector. Long-range PCR allows the amplification of fragments of up to 22 kb from genomic DNA (Cheng *et al*., 1994). Thus, long-range PCR allows the design of the targeting vector to be unconstrained by size limitations necessary to utilize a PCR screening assay.

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