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Use of a Simplified Single-site PCR to Facilitate Cloning of Genomic DNA Sequences Flanking a Transgene Integration Site

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We have used a simplified single-site PCR based strategy to isolate genomic DNA flanking the transgenic insert in a line of transgenic mice. The flanking sequences, which were refractory to more conventional cloning techniques, were isolated and characterized within 1 week. This strategy begins with the annealing and ligation of a single-stranded oligonucleotide to recessed 5′ ends of restriction endonu-clease-digested, size-selected transgenic DNA. Following denaturation, a second oligonucleotide is used to prime DNA synthesis from a position within the transgenic sequences to the end of the genomic restriction fragment, finally synthesizing a complement of the ligated oligonucleotide sequence. Subsequently, a PCR is initiated which uses primers specific for the transgenic DNA and the newly synthesized DNA complementary to the ligated oligonucleotide. The only prerequisite data are sequence information for the transgenic DNA and Southern information regarding the size(s) of restriction fragments that contain the flanking genomic material. This report demonstrates one utility of this technique—enabling rapid isolation of specific mammalian genomic DNA sequences.

Random integration of exogenous DNA into the genome of fertilized mouse oocytes can lead to insertional inactivation of endogenous genes.[1-3] In cases where interesting phenotypes are generated, the isolation of genomic sequences flanking the site of the integrated transgenic complex provides a starting point from which to identify the inactivated gene. Often, this has been accomplished by construction of a genomic DNA library in a bacteriophage λ vector followed by screening of the library using the transgene as a probe.[4-6] However, this approach has at least two drawbacks. First, the preparation and screening of the genomic library is tedious and time consuming. Second, genomic DNA may contain sequences or covalent modifications (e.g., methylation) that render recombinant bacteriophage clones "unstable" in packaging reactions or in host bacteria, thus precluding efficient cloning of certain regions.

Alternative approaches have utilized polymerase chain reaction (PCR)-based methods to facilitate the isolation of DNA adjacent to a region of known sequence identity.[7-18] These PCR strategies can be categorized conceptually into three classes referred to as circular (or inverse) PCR, anchor PCR, and single-site PCR. In circular (or inverse) PCR,[7-9] DNA to be amplified is first digested with a restriction endonuclease, then ligated under conditions that favor recircularization. After nicking of the circular DNA, PCR is performed using primers orientated outward from a region of known sequence identity. With anchor PCR,[10-16] amplification occurs between a known sequence within a DNA molecule and a specific DNA sequence ligated to its termini. The ligated sequence may be a double-stranded DNA linker,[10-13] a homopolynucleotide tract generated by terminal transferase,[14,15] plasmid DNA,[16] or other substrates. For single-site PCR,[17,18] a double-stranded oligonucleotide adaptor is ligated to restriction enzyme-digested or blunt-ended DNA. The double-stranded adaptor is designed such that it contains within it a region of single-stranded DNA, either as 3′ terminal[17] or internal "bubble" mismatches.[18] With either version, the specificity of the PCR is generated by the prerequisite synthesis of DNA complementary to the single-stranded region of the adaptor. The primer for the initial DNA synthesis reaction is an oligonucleotide that binds to a region of known sequence identity within the template DNA. The other primer used in the PCR recognizes only the newly synthesized complementary DNA as a target annealing sequence.

We have been interested in characterizing the recessive *sys* mutation in a line of transgenic mice.[6] Previously, we isolated one flank of the single site of transgene integration in this line of mice by bacteriophage cloning.[6] However, despite screening of four independent bacteriophage libraries constructed with restriction endonuclease-

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digested, size-selected transgenic genomic DNA, we failed to isolate a clone containing the second flank.

Therefore, we attempted to isolate the second flank using a simplification of a single-site PCR technique[17] and compared this directly to an anchor-PCR technique.[16] Although both protocols were successful, the specificity of the simplified single-site PCR appeared greater than that of anchor PCR. In addition to this enhanced specificity, the simplified single-site PCR has advantages over previous single-site PCR methods in that it utilizes a single-stranded oligonucleotide, not double-stranded adaptors or splints, and that the oligonucleotide does not require kinasing prior to use. We describe here the modified technique and its application to the isolation of the transgenic flanking genomic DNA. This method facilitates the rapid isolation of genomic sequences flanking transgene integration sites and any other region of known sequence identity and in particular may assist in the isolation of those mammalian DNA sequences that are recalcitrant to cloning in certain bacterial hosts.

METHODS

DNA Restriction and Modification

DNAs were treated with restriction endonucleases and modification enzymes as described by the manufacturer (CIP, Boehringer Mannheim; *Sst*I, Bethesda Research Laboratories, AmpliTaq, Perkin-Elmer Cetus; T4 DNA ligase and all other enzymes, Pharmacia LKB). The DNA ligation buffer used was that described by Lathe et al.[19]

DNA Isolation and Synthesis

Genomic and plasmid DNAs were isolated as described.[20] Oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer. Following deprotection and lyophilization, oligonucleotides were resuspended in HPLC-grade water and used directly. Genomic DNA from *sys* homozygous mice was digested with *Sst*I and then size-fractionated by electrophoresis through a Tris-acetate EDTA (TAE)-buffered agarose gel (SeaKem, FMC).[20] Digestion with *Sst*I generates a 1.7-kb fragment with the desired transgenic genomic flank. Agarose slices that contained DNA fragments of ~1.4–2.0 kb were generated and the DNA was recovered by spinning through glass wool,[21] followed by precipitation at room temperature in the presence of 2.5 M ammonium acetate and 2.5 volumes of ethanol. A Southern analysis was performed to determine which fractions contained the desired DNA fragment.

Preparation of Single-site PCR Template

Fifty nanograms of *Sst*I-digested genomic DNA was mixed with the "ligation" oligonucleotide (see Fig. 1) in a 10^3-to-1 molar ratio (oligonucleotides to genomic DNA termini) in a final volume of 10 µl. The mixture was heated to 65°C for 15 min (to dissociate any annealed genomic fragments) and then placed directly on ice. Three (Weiss) units of T4 DNA ligase were added and the reaction incubated at 15°C overnight. After raising the reaction volume to 50 µl the reaction was heated to 65°C for 15 min to inactivate the ligase and to dissociate unligated oligonucleotides and then was run through a Sephadex G 50-80 spin column to remove the unligated oligonucleotides.

Preparation of Anchor PCR Template

Ten micrograms of pTZ19r was digested with *Sst*I and the 5′ termini dephosphorylated with CIP. Following phenol extraction and ethanol precipitation,[20] the DNA was resuspended at a concentration of 50 µg/ml. Bacterial transformation was used to determine the efficiency of vector digestion and dephosphorylation. Fifty nanograms of size-selected or total *Sst*I-digested genomic DNA was ligated to the vector in a 1-to-5 molar ratio (insert to vector) to maximize the proportion of insert

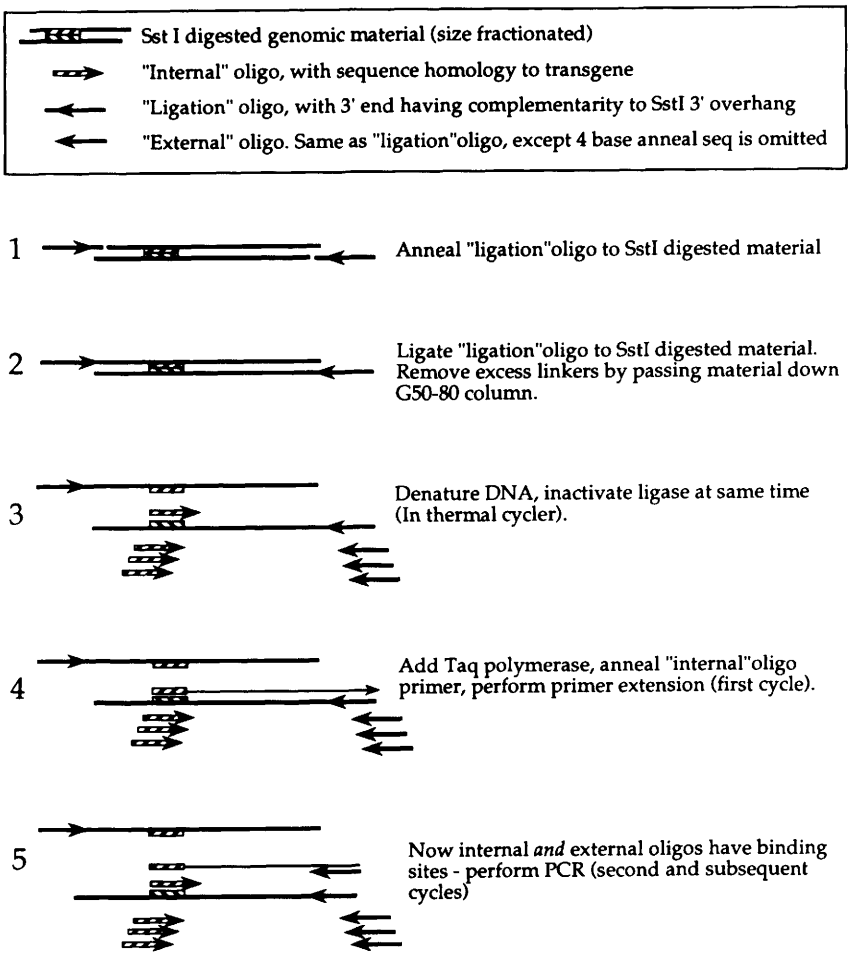

FIGURE 1 Oligo ligation PCR strategy. The boxed section illustrates the components of the reaction. The individual steps of the reaction are illustrated in steps 1–5.

ligated to vector. The ligation was performed overnight at room temperature (27°C) in a final volume of 10 μl using three (Weiss) units of T4 DNA ligase. Before initiating the PCR, the volume was raised to 50 μl and the reaction was heated to 95°C for 30 min to inactivate the ligase and to nick the circular template.[7]

PCR Amplification

PCR was performed using the recommended Cetus buffer system [1x buffer = 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3) at 25°C, 0.01% wt/vol gelatin] in an Ericomp twinblock thermal cycler. Oligonucleotides were used at 500 μM each and deoxynucleotides (dNTPs) at 250 μM each. Five units of AmpliTaq (Perkin-Elmer Cetus) were used in each 100-μl reaction. Total template DNA concentrations in these reactions varied from 250 ng/ml for oligonucleotide ligation to 1 μg/ml for vector ligation. Ten percent (5 μl) of each template was combined with buffer, dNTPs, and the "internal" (specific for the transgenic DNA) and "external" oligonucleotide primers; the mixture was heated to 95°C in the thermal cycler for 10 min to denature the DNA and to inactivate any remaining ligase activity. Following the addition of *Taq* polymerase, the thermal cycler was taken off "hold" and 38 rounds of PCR performed using the following conditions: anneal 58°C, 5 sec; polymerize, 72°C, 2 min 30 sec; denature, 95°C, 30 sec.

PCR Product Recovery and Subcloning

PCR products were recovered by ethanol precipitation. Following digestion of the products with appropriate enzymes, they were subjected to agarose gel electrophoresis, excised from the agarose, and purified by spinning through glass wool.[21] After a further ethanol precipitation, the products were quantified, subcloned into pTZ19r phagemid vector (Pharmacia), and screened using standard techniques.[20]

Southern Analysis

Southern analysis on size-selected and total genomic DNA was performed as described.[20]

Oligonucleotide Primer Sequences

The "internal" oligonucleotide was designed from the sequence of the SV40 genome that is present at the 3′ end of the RSV lacZ transgene.[6] It has 24 bases of homology to SV40 (nucleotide numbers 2295–2319) and a *Hin*dIII site at the 5′ end (plus 4 bases of "buffer" DNA to increase the efficiency of digestion of subsequent PCR products by *Hin*dIII). It has the sequence 5′-ATGCAAGCTTGTAAACAGCCCAC AAATGTCAACA-3′. The "ligation" oligonucleotide, whose 3′ end has complementarity to an *Sst*I 3′ overhang, contains a *Bam*HI recognition site at its 5′ end, and it has the sequence 5′-GGGATCCTGATGCAGTCA GTGCACTACGACAGCT-3′. The "external" oligonucleotide is identical to the "ligation" oligonucleotide, except that the *Sst*I annealing sequence (AGCT) is absent. The oligonucleotide used to amplify sequences cloned into pTZ19r was derived from the polylinker and has the sequence 5′-GCCTGCAGGTCGACTCTAGAGGATC-3′.

RESULTS

A Simplified Single-site PCR

We have simplified a single-site PCR strategy[17] and have used this protocol to isolate genomic DNA flanking the integration site of a transgene complex in the *sys* family of transgenic mice.[6] The protocol is outlined in Figure 1. Initially, genomic DNA is digested with a restriction enzyme that generates a 3′ overhang. Next, the digested DNA is annealed and ligated to a single-stranded oligonucleotide that has complementarity to the 3′ overhang. This generates double-stranded templates with 3′ recessed ends. Following removal of unligated oligonucleotides, the genomic DNA is denatured and annealed to an "internal" oligonucleotide whose target sequence is within the transgene. Next, a primer extension reaction is performed, which terminates after synthesis of DNA complementary to the first, ligated, oligonucleotide. Finally, PCR is performed using a primer specific for the transgene and an "external" oligonucleotide that is identical to the "ligation" oligonucleotide but lacks the region of complementarity to the restriction enzyme digestion site 3′ overhang. As with other single-site PCR strategies, the specificity of this reaction is generated by the "internal" oligonucleotide-mediated primer extension, which synthesizes the template DNA for the other primer in the PCR. The "external" primer should generate no amplification products until DNA complementary to the "ligation" oligonucleotide is synthesized by primer extension from the "internal" oligonucleotide.

Comparison of Simplified Single-site PCR and Anchor PCR in Isolating Specific Genomic DNA Sequences

This simplified single-site PCR technique was compared directly to an anchor-PCR method[16] in their utility to isolate genomic DNA flanking a transgenic integration site. *Sst*I-digested genomic DNA from homozygous *sys* transgenic mice was ligated either to the "ligation" oligonucleotide (see Methods) to generate a single-site PCR template or to pTZ19r to generate an anchor-PCR template. For each strategy, three different genomic DNAs were used: (1) size-fractionated genomic DNA that was positive for the transgenic flank, (2) size-fractionated material that was negative for the transgenic insert (as a negative control), or (3) unfractionated, total genomic DNA. Successful PCR amplification is expected to generate a product of 1.4 kb, this being the distance from the "internal" oligonucleotide annealing site to the *Sst*I genomic site.

Although both strategies resulted in successful amplification of the genomic sequences flanking the transgene (see Fig. 2), use of the size-fractionated genomic DNA with the simplified single-site PCR (lane 1) generated an amplification product of a more specific nature than the equivalent anchor PCR (lane 4). The 1.4-kb species is the predominant reaction product in the single-site PCR; however, there are at least six other species present in the product from the anchor PCR.

With either protocol, the use of total digested genomic DNA as the PCR template failed to generate the specificity achieved with the size-fractionated genomic template. However, again, the simplified single-site PCR strategy succeeded in generating the desired flank-

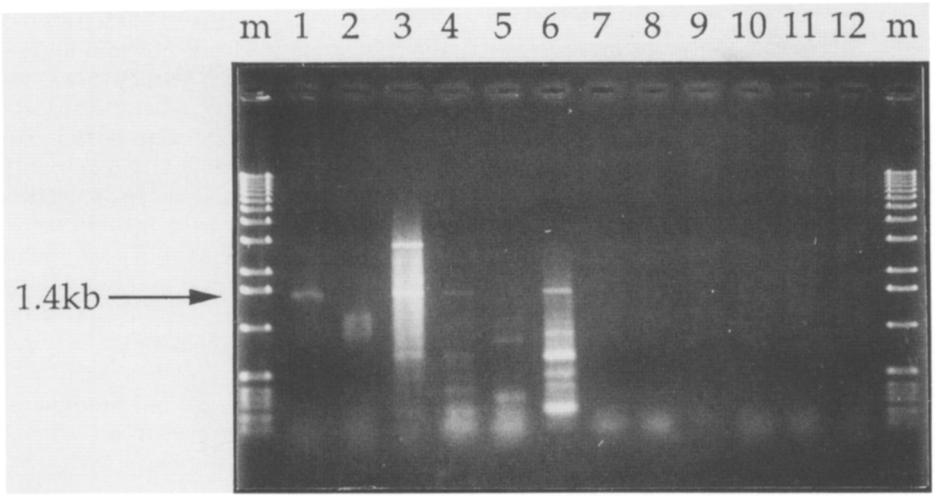

FIGURE 2 Analysis of PCR reaction products. Ten percent of each PCR was run through a 0.7% TAE agarose minigel, stained with ethidium bromide, and photographed on a UV transilluminator. The table below gives information regarding the nature of the genomic DNA and whether the DNA was ligated to the ligation oligonucleotide (O), to pTZ19r vector DNA (V), or unligated (U). "Oligonucleotides" refers to the oligonucleotides used to perform the initial primer extension and to generate the subsequent PCR. (Lanes *M*) Markers, 1-kb ladder (Bethesda Research Laboratories). The arrow indicates the position of the predicted amplification product at 1.4 kb.

Lane	Genomic DNA, *Sst*I-digested	Ligated to	Oligonucleotides
1	size-fractionated, positive for flank	O	internal and external
2	size-fractionated, negative for flank	O	internal and external
3	total	O	internal and external
4	size-fractionated, positive for flank	V	internal and vector
5	size-fractionated, negative for flank	V	internal and vector
6	total	V	internal and vector
7	no genomic DNA, vector only	V	internal and vector
8	total	U	internal and external
9	total	U	internal only
10	total	U	external only
11	total	U	vector only
12	total	U	internal and vector

ing genomic material in appreciable quantities despite a background "smear" of nonspecific amplification products (see 1.4-kb product in Fig. 2, lane 3) whereas the equivalent anchor-PCR reaction could only generate the genomic flank as a minor amplification product (Fig. 2, lane 6). Thus, with either size-selected or total digested genomic DNA as the template, the simplified single-site PCR generated the desired genomic flanking material with greater specificity than the equivalent anchor-PCR reaction.

We have not attempted to determine the nature of the additional, nonspecific PCR products that were generated. They may arise from amplification of the other copies of the RSV-LacZ transgene, as this family has approximately 20 copies of the transgene integrated at a single-site and there are two *Sst*I sites within each transgene.

Size-selected genomic DNA lacking transgene/flanking material did not give an amplification product of 1.4 kb (Fig. 2, lanes 2 and 5). In addition, reactions in which the "ligation" oligonucleotide was not linked to the genomic DNA did not yield an amplification product (Fig. 2, lanes 8–12).

Following an ethanol precipitation, the remaining reactions from lanes 1 and 4 were digested with restriction enzymes whose target sequences had been designed into the oligonucleotides. The single-site PCR material was digested with *Bam*HI and *Hin*dIII, and the anchor-PCR material was digested with *Hin*dIII and *Sal*I. The products were subjected to agarose gel electrophoresis, and the 1.4-kb fragment from each reaction was cloned following gel purification.[21]

Confirmation of Identity of Amplified DNA as Transgenic Integration Site

To confirm the identity of the cloned product as genomic material flanking the transgenic integration site, an RFLP analysis was performed. The results are illustrated in Figure 3. The cloned PCR product was radiolabeled and hybridized to *Sst*I- or *Eco*RI-digested genomic DNA from nontransgenic, hemizygous and homozygous *sys* mice (Fig. 3). The Southern hybridization patterns confirm the identity of the amplified material as genomic DNA flanking the transgene integration site. For *Sst*I-digested genomic DNAs, the probe detects a 1.85-kb band associated with the nontransgenic allele and a 1.7-kb band associated with the transgenic allele. Similarly, for *Eco*RI-digested DNAs, the probe detects a 2.2-kb band for the nontransgenic allele and a 6.4-kb band for the transgenic allele. The probe also hybridizes weakly to a 2.35-kb nonpolymorphic band in the *Eco*RI digests. Identical results were obtained using products from the anchor PCR (data not shown).

DISCUSSION

We have used a simplified single-site PCR technique (see Fig. 1) to isolate genomic DNA sequences flanking the transgenic integration site in a line of transgenic mice. The flanking material had previously proven refractory to isolation in *Escherichia coli*. In this regard, PCR-mediated isolation has dual advantages over more conventional cloning strategies. It is rapid and is unaffected by DNA modifications (e.g., methylation) that can interfere with efficient cloning.

Several PCR-based strategies have been described that facilitate the isolation of DNA adjacent to regions of known sequence identity.[7–18] Of these strategies, for isolation of flanking sequences, single-site PCR has the

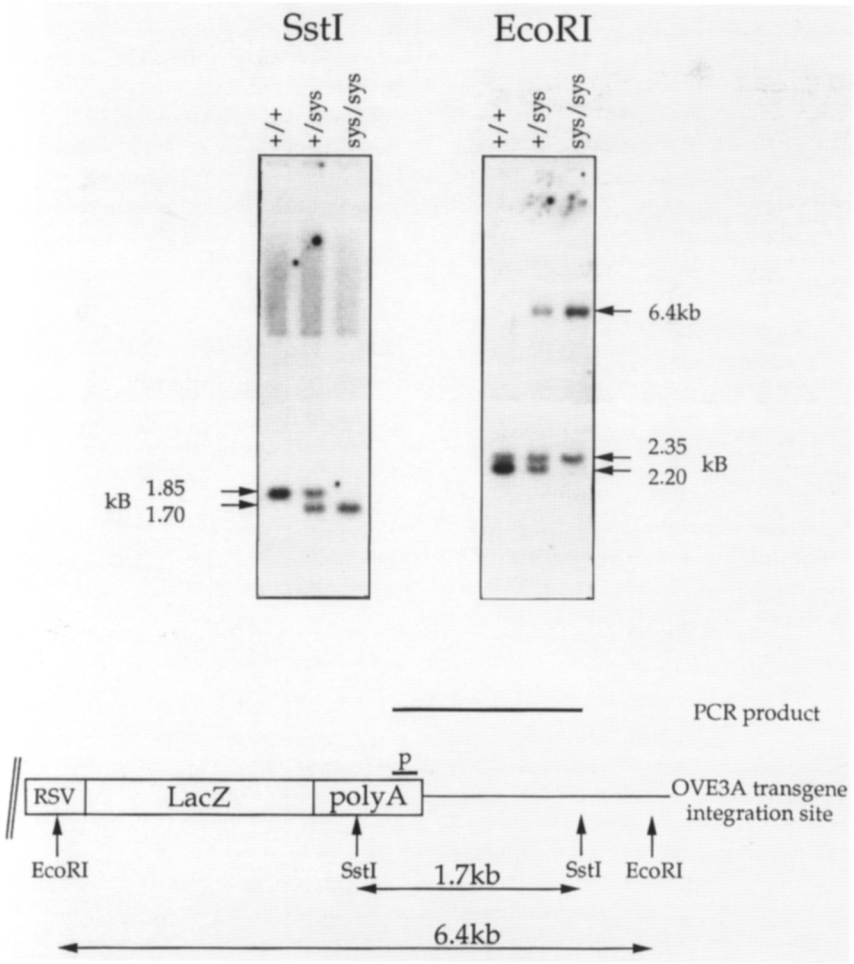

FIGURE 3 Restriction fragment length polymorphism (RFLP) analysis of wild-type and *sys* alleles using a cloned PCR product as a probe. Genomic DNAs from wild-type, heterozygous, and homozygous *sys* mice were digested with *Eco*RI or *Sst*I, subjected to electrophoresis through a TAE agarose gel, and transferred to nylon membranes. These were hybridized with the radiolabeled cloned 1.4-kb PCR product, as illustrated by the schematic. An RFLP is detected for both the *Eco*RI- and *Sst*I-digested material. There is low-copy repetitive DNA that is detected with either probe, as a smear with *Sst*I-digested DNA or resolved by *Eco*RI digestion as a 2.35-kb band. The lower schematic illustrates the 3′ end of the transgene complex including the location of *Sst*I and *Eco*RI restriction sites in the transgene and the flanking genomic DNA sequences. The position of the "internal" oligonucleotide used to perform the initial primer extension is indicated by P.

potential to generate highest product specificity.[17,18] This specificity is achieved by performing a primer extension reaction from a specific site within a DNA fragment that results in synthesis of DNA complementary to a single-stranded region attached to the end of the fragment. It is this specific synthesis that generates the target sequence for a second oligonucleotide in a subsequent PCR.

In previously reported single-site PCR strategies,[17,18] the single-stranded region has been produced by use of duplex adaptor DNA molecules that are ligated to the ends of 5′ overhang, restriction-digested,[17] or blunt-ended[18] template DNA. The adaptors contain a region of mismatch either at the 3′ end of one molecule (resulting in the formation of a "tail"[17]) or within the duplex itself (resulting in the formation of a "bubble" [18]). The presence of this tail or bubble prevents copying of the single-stranded region of the duplex adaptor. Only in the case where DNA synthesis is primed within the denatured template will successful copying of the single-stranded region occur.

Vital to the success of these single-site PCR strategies is the efficient kinasing of the oligonucleotides used to form the adaptor molecules. Failure to ligate the strand of the adaptor that inhibits synthesis of DNA complementary to the single-stranded region of the adaptor will produce template molecules with a 3′ recessed end. Subsequent addition of *Taq* polymerase will result in the synthesis of primer binding sites at both ends of all molecules which could potentially reduce the specificity of a subsequent PCR. However, this problem can be obviated by prior denaturation of the DNA. It follows that no advantage accrues from use of a double-stranded splint or bubble duplex except the ability to ligate these classes of molecules onto DNA digested with restriction enzymes that generate 5′ overhangs or blunt-ended molecules.

We have simplified these single-site PCR methods to utilize a single-stranded oligonucleotide instead of the more cumbersome double-stranded adaptors or splints. This has dual advantages of reduced expense for oligonucleotide synthesis and no requirement for kinasing prior to use, because the 5′ end does not participate in the ligation reaction. Although this single-site PCR strategy requires the digested template DNA to have a 3′ overhang, there are several commercially available restriction endonucleases that generate nonredundant, four-base, 3′ overhangs, e.g., *Aat*II, *Apa*I, *Hae*II, *Kpn*I, *Nla*III, *Nsi*I, *Nsp*HI, *Pst*I, *Sst*I, and *Sph*I. Oligonucleotides can be designed that are complementary to the 3′ overhang of the particular restriction enzyme used.

We performed an experimental comparison of the relative abilities of the simplified single-site PCR and an anchor-PCR method[16] to amplify genomic material flanking a transgene integration site in mouse genomic DNA. Enhanced product specificity (the relative ratio of specific to nonspecific PCR product) was observed experimentally using size-selected genomic DNA as the template when we compared the simplified single-site PCR to the anchor-PCR method (in the latter, specific oligonucleotide priming from an internal sequence is not required to generate the second oligonu-

cleotide binding site). This ability of the simplified single-site reaction to generate a more specific PCR product was also observed when total genomic DNA was used as template, although these reaction specificities were lower than those achieved with size-selected, flank-containing genomic DNA. Overall, the simplified single-site PCR appears superior to the anchor PCR-method.[16] Although a direct comparison with other single-site PCR strategies is difficult to make, the simplified single-site PCR technique does appear to have greater sensitivity and specificity compared to one of its predecessors.[17]

In performing the primer extension step of the simplified single-site PCR, it is important to ensure thorough denaturation of the template. In practice, this can be achieved by placing the thermal cycler on "hold" at 95°C while performing the initial denaturation and, following addition of *Taq* polymerase with the Eppendorf tube in the block, taking the machine off "hold" to start the first cycle. Thorough denaturation will eliminate the synthesis of DNA complementary to the ligated oligonucleotide via extension of 3′ recessed ends of nondenatured template and prohibit the formation of erroneous primer extension products. During the annealing step of the first cycle, the complexity of the genomic mixture is sufficient to preclude synthesis of DNA complementary to the "ligation" oligonucleotide that could occur through reassociation of genomic DNA strands.

Also, it is vital that the highest possible annealing temperature be used to ensure reaction specificity. In our experience, using primers with 25 bases of homology, 45–55% GC content, and the Cetus-recommended buffer system, specificity of annealing was achieved using temperatures between 55°C and 60°C. In theory, with this annealing temperature range, it should be possible to simplify the reaction components further. As the "ligation" oligonucleotide has only 4 bases of homology at its 3′ end to the 3′ ends of the digested genomic DNA, this oligonucleotide could be used to replace the "external" oligonucleotide in the PCR. However, experimentally, all attempts to replace the "external" oligonucleotide in this manner have resulted in drastic loss of specificity of subsequent PCR products (data not shown).

Due to the presence of more than one copy of the transgene in the particular line of mice analyzed, to achieve the highest reaction specificity, it was necessary to gel-purify digested genomic DNA containing the appropriate target sequence prior to ligating the "ligation" oligonucleotide and performing the PCR. (However, amplification of the desired genomic material was achieved, albeit with lower specificity using total digested genomic DNA as the template.) In cases where additional restriction mapping data is available, the specificity of amplification might be enhanced by digesting the template DNA with additional enzyme(s). The additional enzymes would be chosen to restrict annealing of the "ligation" oligonucleotide to a subset of the digested DNA thereby preventing amplification of internal copies of the transgene.

The main advantages of this single-site PCR technique are its simplicity, the minimal reagent requirements, and its ability to generate the desired product even from templates of high complexity such as total digested genomic DNA. It may prove useful not only for this, but for additional problems such as definition of intron–exon boundaries using cDNA sequence information and isolation of retroviral integration sites.

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