Figure 2. Physical map of pCKC1. The map of pCKC1 is shown in the same orientation as pGEM-7Zf(+) from which it was derived. Information on this plasmid is available from Promega. The unique restriction sites in the MCS are shown.

lower right, plasmids A and B). Only intermolecular ligation of the nonmutant molecules can accomplish this and even then, there is only a 50% chance that this rare ligation event will occur in the correct orientation (Figure 1, lower right, plasmid C in correct orientation, plasmid D in incorrect orientation). Hence, the vast majority of nonmutant molecules will not yield plasmids with the CAT gene intact; all of the mutant molecules will. After digestion with *EcoRI*, the sample was diluted to 500 μ L with T4 DNA ligation buffer, and 4 U of T4 DNA ligase were added. The ligation was run at 16°C overnight. The ligation mixture was used to transform *E. coli* strain BMH71-18 *mutS*, and the cells were plated on chloramphenicol-containing L plates. The DNA from the resultant colonies was screened for the presence of a single *EcoRI* site. Approximately 50% of the possible mutant plasmids contained a single *EcoRI* site and were screened further. These plasmids were not cleaved with *PstI*, cleaved once with *SphI*, and cleaved twice with *NaeI* confirming that they had also incorporated the (-)*PstI* mutagenic primer in the initial reactions. This new plasmid is pCKC1 (Figure 2). It is approximately 4 kbp in size, encodes chloramphenicol resistance and has the unique restriction enzyme sites suitable for cloning shown in the figure. Unlike the suggested protocol (3), we only used one round of restriction en-

zyme selection. A second round would increase the probability of isolating a mutant plasmid.

This modification of the U.S.E. protocol does not require the elimination of a unique restriction site. In this case, the unwanted site is in the CAT gene coding region, requiring the intermolecular ligation of two nonmutant fragments in the correct orientation to reconstruct the gene. Diluting the ligation reaction should enhance the frequency of intramolecular relative to intermolecular ligations. Although the elimination of a site within the coding sequence of a selectable marker should further enhance the frequency of obtaining mutants, this is not a necessity. If the two sites in question are far enough separated on the plasmid, and if cleavage of these sites leads to separation of important plasmid functions (such as antibiotic resistance and the origin of replication) the more probable intramolecular circularization of either of the two nonmutant fragments will not be able to produce a viable plasmid.

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Bi-directional Dideoxy Fingerprinting (Bi-ddF): Rapid and Efficient Screening for Mutations in the Big Blue™ Transgenic Mouse Mutation Detection System

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Single-stranded conformation polymorphism (SSCP) (9) is currently the most widely used method for screening for mutations. SSCP does not detect all sequence changes, and its sensitivity depends on the sequence and size of the segment screened (3,6,8,11,12). For a sequence of about 200 bp, the observed sensitivities are often in the range of 60%–90%. For larger segments, the sensitivity is decreased. Dideoxy fingerprinting (ddF) is performed by electrophoresing one lane of a Sanger dideoxy termination reaction through a non-denaturing gel (11). ddF can screen a 250-bp segment with essentially 100% sensitivity (1,6,11). In bi-directional dideoxy fingerprinting (Bi-

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ddF), the cycle sequencing protocol is modified by performing simultaneous Sanger dideoxy termination reactions in the downstream (D) and upstream (U) directions in the presence of only one dideoxy terminator (ddGTP) (Figure 1A). Bi-ddF is a very powerful tool for screening mutations and has been used to screen up to a 577-bp region (over double the size of the segments screened by the original ddF) with 100% sensitivity (5). Mutations found by Bi-ddF can conveniently be sequenced by using the same DNA template and one of the primers for cycle sequencing. Here we have applied Bi-ddF to screen for mutations in the *lacI* gene, which is a target of mutagenesis in the recently developed transgenic mouse mutation detection system (Big Blue™; Stratagene, La Jolla, CA, USA). This blind, prospective analysis with a G+C rich template (55%) provides further evidence for the high sensitivity and specificity as well as the versatility of Bi-ddF.

Big Blue transgenic mice carry chromosomally integrated lambda phage shuttle vectors (LIZ) containing the *E. coli lacI* gene as a target for mutagenesis (4). The lambda vector including the *lacI* gene can be rescued by in vitro packaging and screened for mutations in the *lacI* gene by infecting and plating *E. coli* on a rich medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as the chromogenic substrate (4). Mutant lambda phage, which produced blue plaques, were re-plated at a low density to purify mutant plaques. Blue plaques as well as some colorless plaques were picked carefully to avoid contamination from neighboring plaques, dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), heated in boiling water for 10 min and stored at -20°C until use. Eighteen plaques (17 blue and 1 colorless) were analyzed in a blind fashion by bi-directional ddF (J.H.) and direct cycle sequencing (H.N.).

A 1338-bp segment containing the entire *lacI* gene and *lacZ* operator region was amplified by polymerase chain reaction (PCR) with primers -55D (5' GTACCCGACACCATCGAATG 3') and 1283U (5' GAGT-CACGACGTTGTA 3') using the following conditions: 1.2 μL of sample,

1.5 mM MgCl₂ and 0.1 μM of each oligonucleotide in a 60-μL reaction volume; 35 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 80 s and elongation at 72°C for 2 min, followed by 10 min of extension at 72°C. The PCR product was then purified by using Microcon-100™ ultrafiltration units (Amicon, Beverly, MA, USA) according to the manufacturer's instructions. The purified samples were electrophoresed on an agarose gel along with a known amount (a total of 200 ng) of *Hae*III digests of φX174. The DNA concentration was estimated by comparing the intensity of each band with restriction fragments of φX174. The concentration of the DNA was then adjusted to approximately 20 ng/μL with distilled water.

The following three sets of primers were used to analyze the 1.3-kb segment depicted in Figure 1B. Primers were radiolabeled by the end-labeling technique using [³²P]ATP or [³³P]ATP and T4 polynucleotide kinase as described previously (10). The base numbering system is according to Farabaugh (2).

Primer set 1 to screen region 1 (453 bp between bp -55 and 398): -55D (5' GTACCCGACACCATCGAATG 3'); 398U (5' GGTTCATCCAGCGGATAGTTAAT 3').

Primer set 2 to screen region 2 (473 bp between bp 331 and 803): 331D (5' AAGCGGCGTCGAAGCCTGTAA 3'); 803U (5' AACGCGCAGCCCGGAC-TC 3').

Primer set 3 to screen region 3 (490 bp between bp 743 and 1232): 743D (5' CCCACTGCGATGCTGGTT 3'); 1232U (5' GAATCCGTAATCATGGT-CATA 3').

Bi-directional Sanger dideoxy termination reactions with ddGTP were performed in a volume of 7 μL containing 50 mM KCl, 50 mM Tris-HCl, pH 8.8, 2.5 mM MgCl₂, 20 μM of each dNTP, 100 μM ddGTP, 0.05 μM each of radiolabeled downstream and upstream primers, 0.4 U AmpliTaq® (Perkin-Elmer, Norwalk, CT, USA) and 5–10 ng template DNA. The ratio of downstream and upstream primers was adjusted so that the termination products generated from the upstream and downstream primers were of similar intensity. The cycling conditions were 30

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cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min. For each set of screening reactions, two unidirectional control reactions were performed using wild-type DNA with one labeled and one non-labeled primer. The above three pairs of primers were used to analyze the 1.3-kb segment.

The Bi-ddF reaction was stopped by adding 40 µL of stop/loading buffer (50% formamide, 7 M urea, 2 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol). The sample was denatured by heating to 80°C for 3 min

and electrophoresed on 0.5× MDE™ gel (AT Biochem, Malvern, PA, USA), 50 mM Tris-borate, 1 mM EDTA and 20 mM HEPES, pH 8.0 by using a Pocker Face SE™ 1500 Sequencing apparatus (Hoefer Scientific, San Francisco, CA, USA). The electrophoresis buffer contained 50 mM Tris-borate, 20 mM HEPES and 1 mM EDTA (pH 8.0). The electrophoresis was performed at room temperature, and the plate was cooled by circulating water to maintain the temperature at approximately 20°C. The gel was then dried and subjected to autoradiography. Mutations were de-

tected by comparison of fingerprints from wild-type and unknown DNAs.

The same 1.3-kb segment was sequenced by using Taq DyeDeoxy Terminator Cycle Sequencing Kit™ (Perkin-Elmer/Applied Biosystems Division [PE/ABI], Foster City, CA, USA) and fluorescence-based ABI automated sequencers (Model 373A or

Figure 1. A) Schematic of bi-directional ddF (Bi-ddF). A region of interest is amplified by PCR from genomic DNA with the first set of primers (P₁ and P₂). Using this DNA as template, Sanger dideoxy termination reactions are performed with only ddGTP and a second set of radiolabeled downstream (D) and upstream (U) primers. In this example, one termination segment (open circle and dotted line) is lost due to the mutation (dideoxy component). Additionally, five termination segments (two downstream and three upstream) carry the mutated base (closed circles), which potentially generates an informative SSCP component on a non-denaturing gel. B) Schematic representation of the regions screened and mutations found. The 1.3-kb fragment, including the entire *lacI* gene and *lacZ* operator, was amplified with primers -55D and 1283U. Mutations were screened by Bi-ddF in three overlapping regions (1, 2 and 3). Primers used for Bi-ddF and sequencing are shown above the line. Sites and types of mutations are shown below the line. The horizontal line is not necessarily proportionate to the number of bases. The base numbering is according to Farabaugh (2).

Figure 2. A portion of representative Bi-ddF gel. Every other lane is numbered. Mutations can readily be detected as dideoxy component changes and/or SSCP component changes when fingerprints from unknown samples and wild-type DNA are compared. Such changes are seen by comparison of lanes 5, 6, 14, 16 and 17 with lane 2, the wild-type phage DNA fingerprint. Lanes 15 and 20 contain ddF reactions generated with one each of respective primers using wild-type DNA.

377A) (PE/ABI). By using the three downstream primers, i.e., -55D, 331D and 743D, the entire 1.3-kb fragment was sequenced in each sample. With each primer, approximately a 450-bp segment was sequenced. Each mutation was confirmed by sequencing the same regions in the opposite direction by using one of the three upstream primers, i.e., 398U, 803U or 1232U, for region 1, 2 and 3, respectively. In some samples, primer -17D (5' ATGGCATGATAGCGCC 3') was used to sequence the first region.

Figure 2 shows a representative bi-directional ddF gel. Mutations are readily detected as lanes with abnormal patterns of bands. By comparing the band patterns with the unidirectional upstream and downstream control reactions (lanes 15 and 20), it was generally possible to localize the positions of the individual mutations (± 50 bp). A blind analysis of 18 samples (54 screened regions) was performed (23 kb of total sequence). Single mutations were detected in all except the one colorless, wild-type plaque by both Bi-ddF and direct sequencing (Figure 1B). Based on the results of direct sequencing, 17 of 17 mutations (13 substitutions, 2 deletions and 2 insertions) were detected (100% sensitivity) and 32 of 32 normal regions were reported as wild-type (100% specificity) by Bi-ddF. Five mutations were located in the overlapping regions and were detected twice as mutant. Within the limit of the small sample size, the sensitivity and specificity of Bi-ddF was 100% (54 out of 54). No false-positive or false-negative results were obtained for this group of samples.

Bi-ddF is simple to perform and analyze. Mutations are detected as dideoxy component changes (loss or gain of a ddGTP band) or as SSCP component changes including a change in position, intensity or width of any band (5). Dideoxy and SSCP components from both strands contribute to the sensitivity of Bi-ddF (Figure 1A). If ddGTP is used in the termination reaction, a termination segment is gained when A, T or C is mutated to G, or lost if G is mutated to A, T or C (Figure 1A). If the Sanger termination reactions in the downstream and upstream directions are considered, nearly 50% of the mutations are associated with a gain of

a segment and 50% with a loss of a segment. An A→T or T→A transversion will not have an informative dideoxy component. Thus, 10 of 12 (83.3%) types of potential single-base substitutions will be detected as a loss or gain of dideoxy components. In Bi-ddF, the SSCP component can be informative for the downstream and/or the upstream direction, which complement each other

to increase the sensitivity (Figure 1). If one or more segments display an altered mobility, the SSCP component is considered informative. Typically, the limit of resolution is a migration change of 1/2 band width on the upper part of the gel and a change of 1/4 band width on the lower part (5). Apparently one normal segment may occasionally be composed of two or more partially

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or completely overlapping segments. If these segments are resolved into separate segments, the segments are scored as positive. Furthermore, small procedural differences may make a difference. For optimal results, readers are referred to the helpful hints published elsewhere (1,5–7). Briefly, some of the technical tips in Bi-ddF are as follows: (i) Suboptimal extension reactions may give rise to shadow bands that can mimic altered patterns due to mutation; (ii) the ratio of the downstream and upstream primer concentrations needs to be optimized so that the termination products generated from upstream and downstream primers are of similar intensity; (iii) the ratio of ddGTP to dGTP needs to be optimized; and (iv) radiolabeling with ^{33}P instead of ^{32}P reduces the thickness of the segments and increases the sensitivity. Lastly, in principle, this technique can be modified for use with fluorescent dye primers or terminators, e.g., by using different dyes for the upstream and downstream primers.

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