Chemiluminescent Detection of Unique Sequences on Chromosomes After On-Slide PCR

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We have recently developed a technique that allows the physical mapping of DNA sequences as short as 100 bp (10,11). Direct in situ single-copy (DISC) polymerase chain reaction (PCR) involves PCR carried out directly on metaphase chromosomes, with incorporation of biotin-11-dUTP into the PCR product followed by an avidin-peroxidase-diaminobenzidine detection system. We have been using this methodology to physically assign extremely short single-copy Type I (coding) and Type II (polymorphic) sequences to chromosomal positions. However, signal intensity is low after using this chromogenic detection system.

Chemiluminescence is a light-emitting chemical reaction. Chemical energy, generated by the decomposition of a weak bond, produces intermediates in an excited state that decay to a ground state with light emission (5). It has been extensively used in protein blotting (8) and DNA probe assays (1,2) because of its high sensitivity, speed and nonhazardous attributes (7). The breakthrough of chemiluminescence in the field of solution immunoassay and transfer membranes prompted us to explore whether this detection system would be more sensitive than chromogenic reporters for detecting PCR products at specific sites on porcine chromosomes. Because the amplified region is usually extremely short (100–400 bp), we reasoned that after DISC-PCR it might be possible to generate a discrete, strong signal that could be assigned to a specific chromosomal position.

We report here the use of chemiluminescence to detect expressed sequence tagged (EST) sites on metaphase chromosomes after the DISC-PCR. Primers for a short fragment of the porcine α interferon (INFα) gene were utilized for DISC-PCR, with incorporation of biotin-11-dUTP followed by chemiluminescent detection. INFα has already been mapped to porcine chromosome 1q by chromogenic DISC-PCR (11) as well as by isotopic (12) and non-isotopic (9) in situ hybridization.

Porcine metaphase chromosomes were prepared from phytohemagglutinin (PHA)-stimulated lymphocytes, and the DISC-PCR was carried out as previously reported (11). The target sequence of the amplified INFα gene was 343 bp.

Following the DISC-PCR, the slides were allowed to cool to room temperature, the coverslips were removed, and the slides were immersed in 4× standard saline citrate (SSC) for 10 min, then in phosphate-buffered saline (PBS) for 2 min. Each slide was then treated with 150 µL streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) at a concentration of 5 µg/mL in detection buffer (1× PBS, 1% BSA, 5 mM EDTA), covered with a 24- × 50-mm coverslip and incubated at 37°C for 1 h in a high-humidity chamber. The slides were washed twice for 10 min in 1× PBS at room temperature, blocked with goat serum–detection buffer (detection buffer plus 5% normal goat serum; Sigma Chemical, St. Louis, MO, USA) for 5 min, then treated with 150 µL biotinylated anti-streptavidin (Vector Laboratories) at a concentration of 2.5 µg/mL in goat

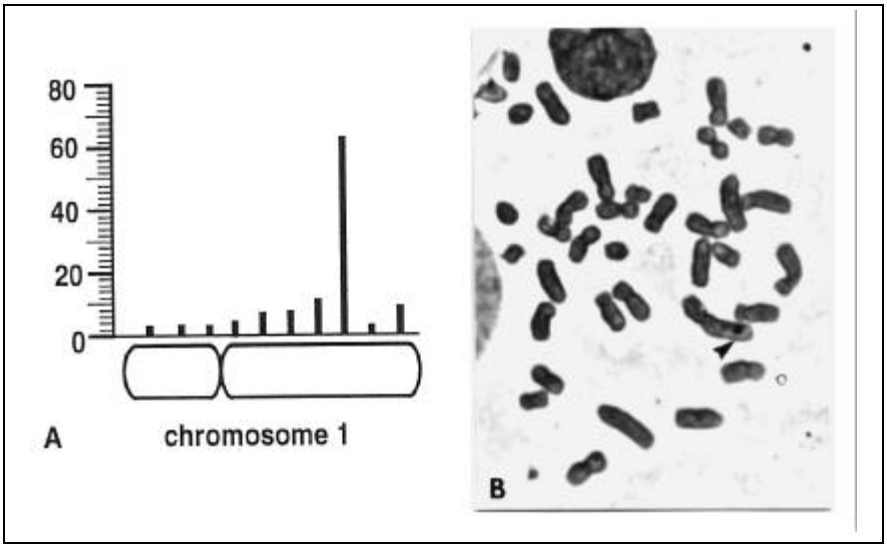

Figure 1. Localization of α interferon gene to porcine chromosome 1. (A) Diagrammatic representation of the signal distribution on porcine chromosome 1 for INFα by chemiluminescent detection. A significant peak was observed at FLpter 0.75–0.76. (B) A partial porcine metaphase spread after chemiluminescent detection of DISC-PCR-amplified IFNα. The arrowhead indicates silver grains.

serum–detection buffer, covered and incubated as above. Slides were then washed twice in PBS and treated with streptavidin-horseradish peroxidase as described above.

LumiGLO™ (Kirkgaard & Perry Laboratories, Gaithersburg, MD, USA) was used as the substrate for horseradish peroxidase as directed by the manufacturer. Equal parts of solutions A and B were mixed, and 50 µL were added to the slides and coverslipped. After incubation at room temperature for 5 min, the coverslips were removed and as much excess solution as possible was shaken off. The slides were carefully dipped in complete darkness in melted (45°C) photoemulsion (Eastman Kodak, New Haven, CT, USA) diluted 1:1 with water. Excess photoemulsion was removed from the backs and ends of the slides, and they were placed in a light-tight box for 2 h. The slides were developed in complete darkness without agitation in D19 developer (Eastman Kodak) diluted 1:1 with water and held at approximately 15°C for 4 min, then dipped in water for 30 s and fixed with regular fixer for 5 min. The slides were rinsed thoroughly by transferring them to a clean container of distilled water every 30 s for a total of 5 min. They were allowed to dry thoroughly in a dust-free area and counterstained with 3% Giemsa for 5 min.

Signals were visualized as silver grains deposited on metaphase chromosomes (Figure 1B). Bright-field photomicrographs were taken with an Olympus BH microscope (Lake Success, NY, USA) (100× objective, oil immersion) and Kodak technical Pan film.

There were 258 total grains counted, 113 of which were localized to chromosome 1. Of these, 63 (56%) were found at FLpter (fractional length of the chromosome with reference point pter) (6) 0.75–0.76 (Figure 1A), which is compatible with the assignment by chromogenic DISC-PCR. Using the Z_{max} test (3), the accumulation of grains in this region is statistically significant (P <0.01).

To our knowledge this is the first report of the use of detection chemistry based upon chemiluminescence to assign loci to metaphase chromosomes. This detection system is considerably more sensitive than enzymatic signaling by means of a chromogenic substrate. However, the major disadvantage is that precise localization of silver grains can be somewhat problematic due to the layers of emulsion film. Nonetheless, because of the increase in sensitivity, this detection method should facilitate the use of DISC-PCR to directly chromosomally assign extremely short single-copy sequences. It should also improve visualization of loci on chromosomes after the primed in situ labeling (PRINS) (4) reaction.

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PCR-TIES (Third Irrelevant Enzyme Site)-Mediated Gene Fusion Method

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There are many advantages associated with the development of bifunctional fusion proteins in molecular biology and for gene therapy. Examples of these include β-galactosidase–*neo*^{r}, β-geo (4); *hyg*^{r}–HSV thymidine kinase, HYTK (6); and β-gal–HSVtk, GALTEK (7). The conventional method to generate fusion genes uses unique restriction sites near the ends of both genes (8). This method depends wholly on the fortuitous location and compati-

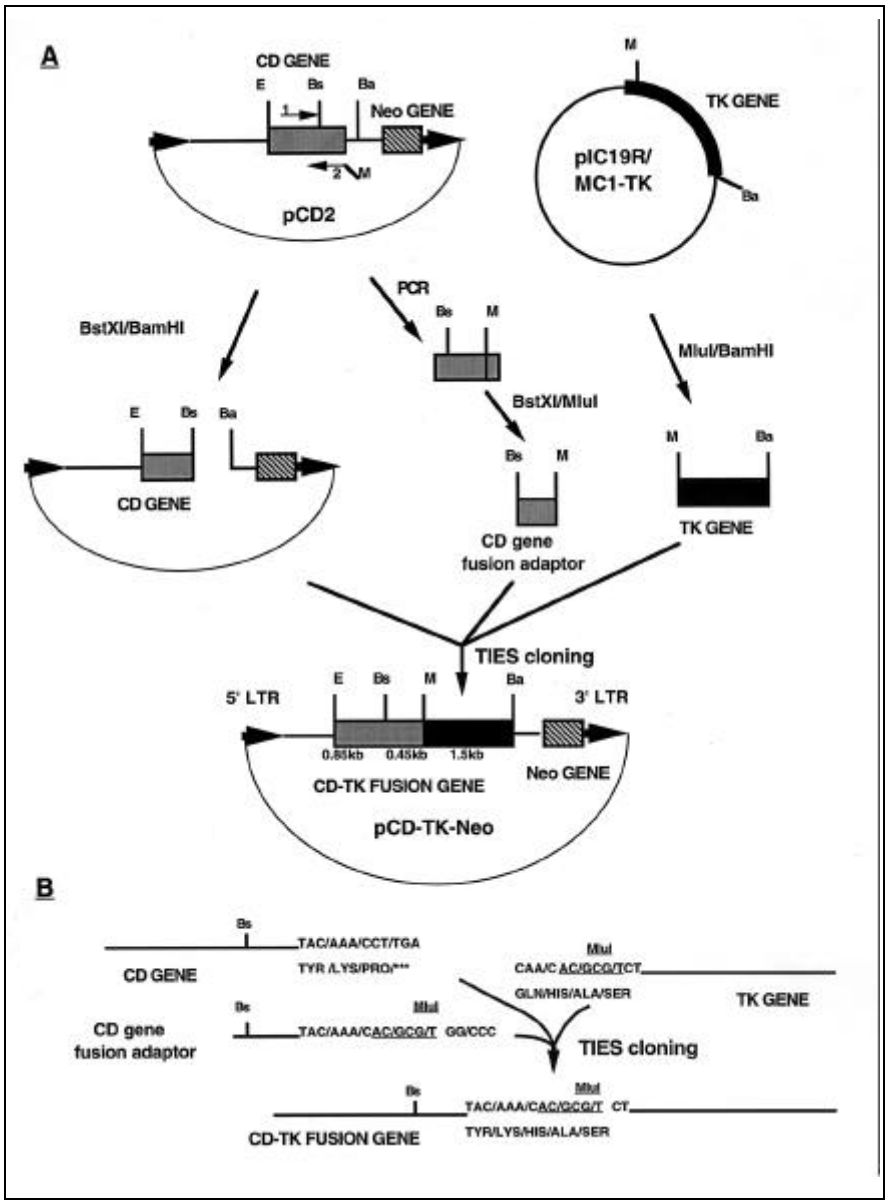

Figure 1. Construction of a fusion gene using the PCR-TIES method. Panel A: The *Mlu*I site at a position 26 nucleotides (nt) from the 5′-end of the HSVtk gene was selected as the fusion enzyme site from pIC19R/MC1-TK. Upstream primer 1, 30 bp 5′ to the *Bst*XI site (5′CACGCCTGTTCCGCTTGCTG3′ [from 649 nt to 668 nt]), and downstream primer 2, with a 5-bp tail after the *Mlu*I site (5′GGGCC(A/CGC/GT)G/TTT/GTA/ATC/GAT/GG/CTT/C3′) (1051 nt - 1069 nt plus the *Mlu*I site and
TK codon CD codon
tail) were used for amplifying a PCR adaptor fragment from the CD gene in a 100-µL volume with 200 µM dNTP, 1.5 mM $MgCl_2$, 100 pmol of each primer, 1 ng template (pCD2 plasmid), 1× reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton® X-100) and 2.5 units of *Tth* DNA polymerase (Promega, Madison, WI, USA). Cycle conditions were 95°C for 1.5 min, followed by 30 cycles of 94°C denaturation for 1 min, 55°C annealing for 1 min and 72°C extension for 1 min, and an additional 10-min extension at 72°C after the last cycle. The PCR product was purified by MicroSpin™ column S-300 (Pharmacia Biotech, Piscataway, NJ, USA) and digested by *Bst*XI (Bs) and *Mlu*I (M). Plasmid pCD2 was digested with *Bst*XI and *Bam*HI (Ba) to generate the CD gene backbone. The HSVtk plasmid was digested with *Mlu*I and *Bam*HI to release the tk gene. The adaptor (0.2 µg) tk fragment (0.4 µg) and pCD2 backbone (1.0 µg) were ligated in 20 µL with 1 µL of 6 Weiss units/µL T4 DNA ligase (New England Biolabs, Beverly, MA, USA) at 16°C overnight and transformed into XL_2-Blue cells (Stratagene, La Jolla, CA, USA) as described in Reference 9. Panel B: Detail of the junction. This diagram shows the relationship at the junction point of the fusion gene, CD adaptor and tk gene. Note that the junction sequence comes directly from the fusion-enzyme primer sequence, so that the junction point can be made to be located at any codon within the adaptor fragment of the CD gene with a guarantee of in-frame fusion.