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Intra-strain polymorphisms are detected but no genomic alteration is found in cloned mice ☆

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Abstract

In-gel competitive reassociation (IGCR) is a method for differential subtraction of polymorphic (RFLP) DNA fragments between two DNA samples of interest without probes or specific sequence information. Here, we applied the IGCR procedure to two cloned mice derived from an F1 hybrid of the C57BL/6Cr and DBA/2 strains, in order to investigate the possibility of genomic alteration in the cloned mouse genomes. Each of the five of the genomic alterations we detected between the two cloned mice corresponded to the 'intra-strain' polymorphisms in the C57BL/6Cr and DBA/2 mouse strains. Our result suggests that no severe aberration of genome sequences occurs due to somatic cell nuclear transfer.

Keywords: Mouse; Polymorphism; IGCR; Subtraction

Since the sheep 'Dolly' was successfully cloned by somatic cell nuclear transfer (SCNT) [1], the inefficiency of SCNT cloning and abnormality of cloned animals have been a focus of considerable controversy [2]. In the case of mice, the efficiency of SCNT cloning is only 0.2–5.8%, and various abnormalities such as placentomegaly, immune impairment, and obesity are often observed [3]. These problems are particularly important in the assessment of the potential therapeutic applications, and also of academic interest.

To address this issue, many studies have been conducted that examine the epigenetic differences between cloned animals, such as the disruption of imprinted gene methylation [4] and aberrant gene expression patterns [5]. In contrast, the evidence for genetic differences between normal and cloned animals is scant. In this paper, we applied the in-gel competitive reassociation (IGCR) procedure to cloned mice genomes in an attempt to identify genetic alteration in cloned mice. IGCR is a method to enrich unknown, subtle DNA differences—such as small insertions and deletions—between two DNA samples without probes or specific sequence information [6,7]. We detected five mutations between two cloned mice and all five sequence differences were identified as 'intra-strain' polymorphisms in the C57/BL6 and DBA/2 mouse strains. This result suggests that no severe aberration of genome sequences occurs due to somatic cell nuclear transfer.

Materials and methods

We applied the IGCR procedure in two combinations; one using cloned mouse1121 as the target and the other with cloned mouse1126 as the target. A control subtraction was also used in each experimental IGCR. Although we performed a total of four IGCR reactions, the methods section below mainly describes the IGCR subtraction procedure using 1121 as the target DNA.

The first round of IGCR. Target and reference DNAs were digested with *Sau*3A I. The reference DNA (20 μg) was further treated with bacterial alkaline phosphatase (BAP, 5.6 U) in a 400 μl reaction mixture, which contained 150 mM Tris–HCl (pH 8.0) and 10 mM $MgCl_2$, at 56 °C

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for 1 h. The resulting solution was then phenol-extracted and the DNA precipitated with ethanol. To ensure complete dephosphorylation, the dephosphorylation step was repeated once more. The target DNA digests (200 ng of *Sau*3A I-treated 1121 cloned mouse DNA) were mixed with excess amounts of reference DNA digests (10 μg of *Sau*3A I- and BAP-treated 1126 cloned mouse DNA) and electrophoresed on a 7% polyacrylamide gel (17.6 cm gel length, PROTEAN II xi Cell, Bio-Rad) at 100 V for 17 h in TAE buffer. The portion of the gel containing approximately 0.2- to 1.0-kb DNA fragments (approximately three quarters from the bottom of the gel) was excised, sealed in a hybridization bag, and soaked twice for 45 min in 10 ml of DNA denaturation buffer (0.5 M NaOH, 0.9 M NaCl) at 45 °C. The gel was then incubated four times for 20 min at 45 °C in 10 ml of reassociation buffer (50% formamide, 50 mM Tris–HCl, pH 8.0, 0.9 M NaCl, 10 mM EDTA, and 10% polyethylene glycol 8000) and for an additional 74 h at 45 °C in a hybridization bag. The DNA was recovered from the gel by the "crush and soak" method [8]. The gel was mixed with 4.5 ml of an elution buffer (0.5 M CH_3COONH_4, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.0, and 0.1% SDS), sealed in a hybridization bag, and the gel-crushed and incubated for 16 h at 37 °C. Next, DNA was phenol-extracted and precipitated using ethanol from the elution buffer collected by Ultrafree-MC (Millipore). The DNA (approximately 1.5 μg) was ligated to a *Sau*3A I adaptor R (45 pmol, prepared by annealing synthetic oligonucleotides 5′-AGCACTCTCCAGCCTCT CACCGAG-3′ and 5′-GATCCTCGGTGA-3′) and half of the sample was subjected to PCR-amplification with 50 pmol of primer R (5′-AGCACTCTCCAGCCTCTCACCGAG-3′) in a 50 μl reaction mixture, using the following cycling conditions: 72 °C for 5 min, then 15 amplification cycles (94 °C for 20 s, 68 °C for 10 min), followed by a final extension at 68 °C for 10 min. The PCR product (2 μl) was subjected to a second round of PCR (8 cycles).

Control IGCR was also performed under the same conditions using 1126 DNA as both target (*Sau*3A I-digested) and reference (*Sau*3A I-digested and BAP-treated) DNA to produce a control DNA library.

The second round of IGCR. The PCR products from the experimental and control IGCR were digested with *Sau*3A I (to produce cohesive ends). The PCR product from the control IGCR was digested with *Dpn* I

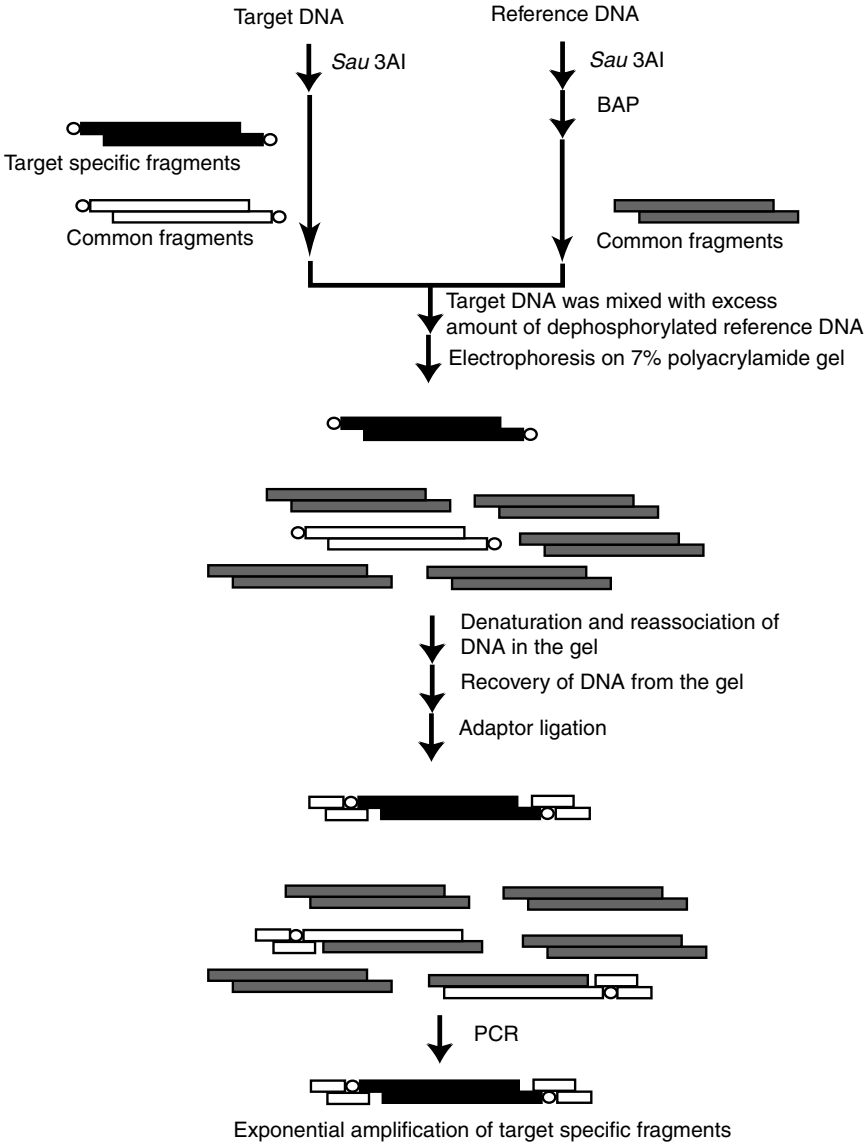

Fig. 1. Schematic diagram of the first round of IGCR. IGCR is a method to enrich target-specific, polymorphic DNA fragments by hybridizing common DNA fragments with non-clonable reference DNA. Closed bars (▬), polymorphic DNA fragments derived from target DNA; open bars (▭), DNA fragments commonly present but derived from target DNA; shaded bars (▬), DNA fragments commonly present but derived from reference DNA, and short open bars (▭), *Sau*3A I adaptor R for PCR amplification. Circles at the end of the bars represent phosphate moieties.

(to produce blunt ends) in the presence of dam methylase and *S*-adenosylmethionine, and the DNA was purified by Montage-PCR (Millipore) to remove adaptor sequences. DNA was then dephosphorylated with BAP. The *Sau*3A I-digested experimental IGCR (40 ng) was mixed with excess amounts (4 μg) of *Dpn* I/BAP-treated control IGCR, and also electrophoresed on a 7% polyacrylamide gel (17.6 cm of gel length, PROTEAN II xi Cell, Bio-Rad) at 100 V for 17 h in TAE buffer. Denaturation, renaturation, and recovery of DNA was performed as described above for the first round, and the DNA (approximately 1.5 μg) was ligated to the other *Sau*3A I adaptor B (45 pmol, prepared by annealing synthetic oligonucleotides 5′-AAAAAACGCCGGCGTACGCGAG-3′ and 5′-GATCCTC GCGTAC-3′). The PCR was performed as described above for the first round of IGCR.

The second round of control IGCR was also performed under the same conditions using *Sau*3A I-digested control IGCR (40 ng) and *Dpn* I/BAP-treated control IGCR DNA (4 μg) from the first round.

The third round of IGCR. The third round of experimental and control IGCR was performed under the same conditions as the second round, using *Sau*3A I-digested DNA (13 ng) and *Dpn* I/BAP-treated DNA (4 μg). The R adaptor and primer were used.

The fourth round of IGCR. The fourth round of experimental and control IGCR was performed under the same conditions as the second round, using *Sau*3A I-digested DNA (2 ng) and *Dpn* I/BAP-treated DNA (4 μg). The B adaptor and primer were used.

Differential hybridization. DNA fragments from the fourth round of the experimental IGCR DNA library (see Fig. 1) were cloned into pBluescript vector. Next, approximately 3000 white colonies were randomly selected and amplified by PCR using the universal T3 and T7 primers of the plasmid vector. The PCR-amplified insert DNA was spotted on nylon membrane (Hybond-N+, GE) using a macroarray spotter (MicroGrid II, Biorobotics), and subjected to differential hybridization using the experimental and control IGCR libraries from the fourth round as probes in order to screen clones that were enriched in the experimental IGCR library. Control and experimental DNA samples (25 ng) were labeled with [α-^{32}P]dCTP (GE, 3000 Ci/mmol) and purified through a G-50 spin column (GE). The ^{32}P-labeled DNA was heated (95 °C for 5 min) and mixed with 10 ml of hybridization buffer (0.5 M Na_2HPO_4, pH 7.2, 1 mM EDTA, and 7% SDS) and was used for the hybridization (65 °C for 16 h). After hybridization, the filters were washed twice with 40 mM Na_2HPO_4 (pH 7.2), 1 mM EDTA, and 1% SDS at 65 °C for 15 min, and once with 0.1× SSC, 0.2% SDS at 65 °C for 30 min. The filters were exposed to an imaging plate (BAS2000, Fuji Film, Japan) for the detection of hybridized signals.

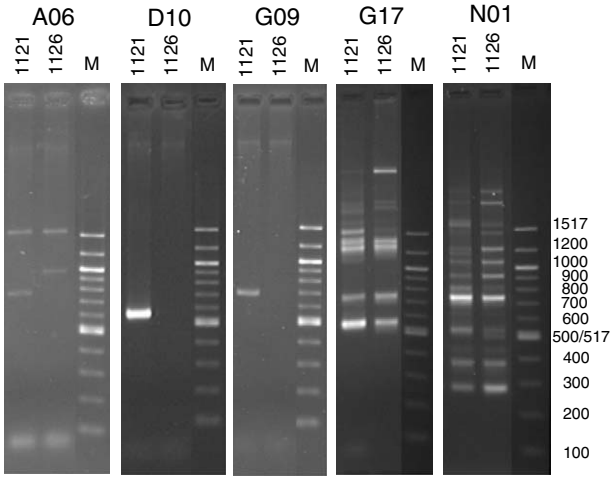

Fig. 2. Obtained clones that show different PCR patterns. Each panel shows the electrophoretic pattern of the PCR product of each clone using the genomic DNA of 1121 and 1126 cloned mice as templates. Lane M is the molecular weight marker.

Results and discussion

We used two cloned mice named 1121 and 1126 from their birth date. The cloned mice were derived from B6D2F1 (C57BL/6Cr × DBA/2) and were cloned using a somatic cell cloning procedure as described previously [9]. In order to enrich the restriction fragment length polymorphism (RFLP) fragments between the two cloned mice, we applied the in-gel competitive reassociation (IGCR) procedure using genomic DNA from the cloned mice (Fig. 1). The subtraction was performed using the two cloned mice in both the target/reference combinations, that is, two patterns of IGCR (both 1121 and 1126 DNA were used as target DNA, together with each control IGCR, see Materials and methods) were performed. After four rounds of IGCR, DNA fragments in the experimental IGCR DNA were

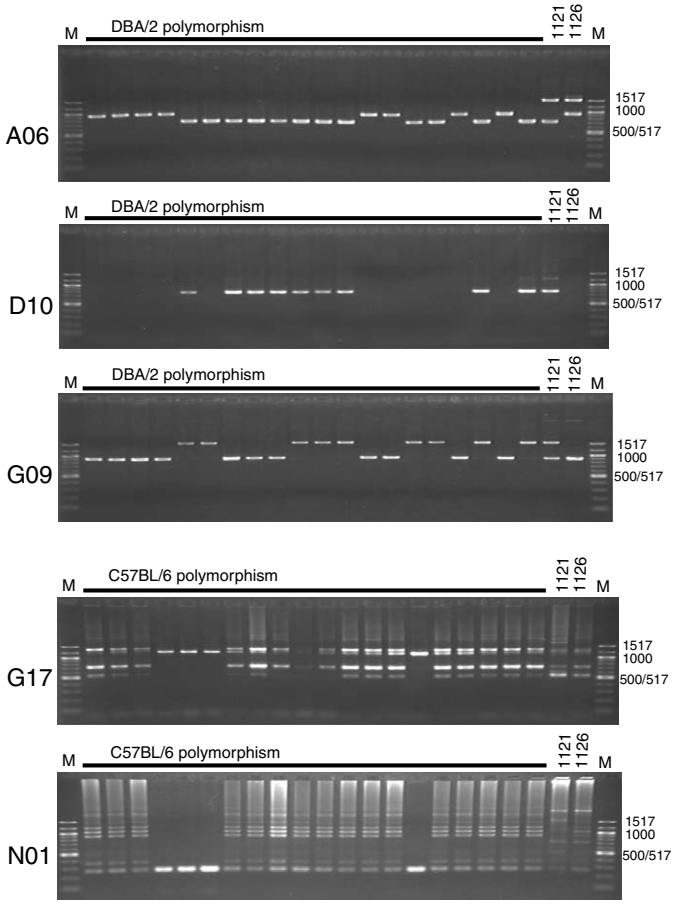

Fig. 3. Characterization of intra-strain polymorphisms. The genomic region of the clones A06, D10, and G09 was amplified by PCR using 20 DBA/2 mice and no difference was observed in C57BL/6Cr (data not shown). The primer sequences of these clones have been deposited with the DDBJ/EMBL/GenBank Data Libraries under Accession Nos. AB244595 (A06), AB244596 (D10), and AB244597 (G09). The genomic region of the clones G17 and N01 was amplified by PCR using 20 C57BL/6Cr mice and no difference was observed between the DBA/2 strains (data not shown). G17 primers, 5′-CTCCCTACCTTCTCTGCCTCATGG-3′ (G17_F3) and 5′-AAAATCCAAAAAGAAAGAAACAGG-3′ (G17_R3); N01 primers, 5′-CCGGATTTTTACCAGGGGAACTTG-3′ (N01_F2) and 5′-ATAC AAAAAGAAAGAAACAGGCAG-3′ (N01_R2).

cloned into pBluescript vector. We randomly selected approximately 3000 colonies from each of the experimental IGCR genomic DNA samples and subjected them to differential hybridization using experimental and control IGCR DNA libraries as probes to screen for enriched clones. We sequenced the enriched clones and designed PCR primers outside and inside of these clones in order to confirm the sequence differences of the cloned mice. After extensive analysis of the enriched clones, we obtained five types of clones that showed different genomic PCR patterns between the 1121 and 1126 cloned mice; four clones (D10, G09, G17, and N01) from 1121-targeted IGCR library and one clone (A06) from the 1126-targeted IGCR library (Fig. 2).

Next, in order to confirm which PCR pattern of these mice was the original B6D2F1 (C57BL/6Cr × DBA/2) mouse pattern, we designed PCR primers to amplify the corresponding genomic regions of these clones from both the C57BL/6Cr and DBA/2 inbred mouse DNA (Fig. 3). The results reveal that all the clones that showed different PCR patterns between 1121- and 1126-cloned mice were derived from the 'intra-strain' polymorphisms in the mouse C57BL/6Cr and DBA/2 strains. The clones A06, D10, and G09 were DBA/2 intra-strain polymorphisms (Fig. 3) and no difference in electrophoretic patterns between the genomic PCR products was observed in C57BL/6Cr strains (data not shown). The clones G17 and N01 were C57BL/6Cr intra-strain polymorphisms (Fig. 3) and no difference was observed among the DBA/2 strains (data not shown). The sequence data of these clones have been deposited with the DDBJ/EMBL/GenBank Data Libraries under Accession Nos. AB244595 (A06), AB244596 (D10), and AB244597 (G09).

From the original library of 3000 colonies, 24 corresponded to the D10 clone, 8 to G09, 17 to G17, 4 to N01, and 11 to A06. This result indicates that additional polymorphic (or mutated) clones cannot be obtained from this library. The polymorphic patterns of clones G17 and N01 appeared to be very similar and the database-matched (only approximately 90% homology) chromosomal region of these clones is very close. We were unable to design primers that amplified the genomic region containing both the G17 and N01 regions, probably due to repetitive-like sequences within these clones. However, it is possible that G17 and N01 were derived from the same region of an intra-strain polymorphic site.

In conclusion, we detected five genomic alterations between two cloned mice and all five sequence differences were identified as 'intra-strain' polymorphisms in the C57/BL6 and DBA/2 mouse strains. Although no information regarding the frequency of intra-strain polymorphisms is available, our results show that no severe aberration of genome sequences occurs due to somatic cell nuclear transfer.

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