2.4. Cryopreservation of spermatozoa

Spermatozoa were frozen and thawed using a slight modification of the method developed by Nakagata and Takeshima [4]. The cryoprotective additives (CPA) consisted of 18% raffinose (Difco, Voigt Global Distribution LLC, Kansas City, MO, USA) and 3% skim milk (skim milk dehydrated; Difco), which were dissolved in MilliQ water at 60 °C. The solution was centrifuged at 20,000 × *g* for 15 min at room temperature; the supernatant was passed through a filter (0.45-μm pore size) and used as CPA. Aliquots of CPA (700 μL each in a 1.5 mL Eppendorf tube) could be stored at −40 °C for up to 6 months. Immediately before use, an aliquot of CPA was thawed and centrifuged at 20,000 × *g* for 15 min at room temperature, and the supernatant was retrieved. Fat and blood were removed from the cauda epididymis using filter paper. Approximately 10 epididymal incisions were made with fine scissors under 100 μL of CPA in a four-well plastic dish (no. 176740, Nunc, Roskilde, Denmark). The spermatozoa were dispersed by gentle shaking of the dish for 1 min, and the sperm suspension was divided into eight aliquots (10 μL each). Each aliquot was placed inside a 0.25-mL plastic straw (Cassou straw; IMV Technologies, L'Aigle cedex, France) by sequentially aspirating about 100 μL of PB1 (~8 cm), air (~2 cm), the sperm suspension (~0.8 cm), air (~2 cm), and PB1 (~1 cm) into the straw. The straw ends were sealed with straw powder (FHK straw powder; Fujihira Industry Co., Tokyo, Japan). The straws were cooled in a freezing canister (50-mL plastic syringe) floating on liquid nitrogen for 10 min, and then immersed directly into liquid nitrogen. For thawing, the straws were removed from the liquid nitrogen and immersed in a water bath at 37 °C for 15 min. The thawed sperm suspension was added to 450 μL of HTF and incubated for 1–2 h at 37 °C. Motile spermatozoa moved into the periphery of the wide shallow HTF drop, while non-motile spermatozoa remained in the middle. Motile spermatozoa were collected as gently as possible with a thin pipette, so as not to affect their motility. They were used for insemination at concentrations of ~50,000 spermatozoa per 20 oocytes.

2.5. Assessment of sperm motility

The motility and the progressive movement of fresh spermatozoa after pre-incubation were assessed using a Hamilton Thorn IVOS computerized semen analyzer (Hamilton Thorn, Beverly, MA, USA). Motility was defined as any movement of the sperm head, and progressive motility was defined as the count of those spermatozoa that moved in a forward, linear direction at a speed of 50 μm/s. Parameters were measured for 100–200 spermatozoa in three different fields. The results were analyzed by ANOVA as described below. Correlations (Pearson's correlation coefficient) between sperm motility and rates of fertilization were also calculated.

2.6. Statistical analysis

The results of the IVF experiments and sperm motility tests were analyzed using arcsine transformation, followed by two-way or three-way ANOVA analysis, as appropriate. A P value of <0.05 for the effects of factors (strain, temperature, and storage days) or

Table 1
Rates of fertilization using mouse spermatozoa retrieved from epididymides stored at low temperatures

Strain	No storage	Storage temperature (°C)	Days of storage			
			1	2	3	4
B6D2F1	284/314 (90)	5	245/308 (80)	266/364 (73)	78/156 (50)	68/146 (47)
		7	237/257 (92)	157/202 (78)	75/149 (50)	76/215 (35)
B6	245/256 (96)	5	144/336 (43)	174/297 (59)	68/234 (29)	59/338 (18)
		7	237/400 (59)	208/295 (71)	146/255 (57)	77/254 (30)

Results from 2 or 3* replicate experiments. Values in parentheses are in percent (%).

interactions between factors was considered significant. When any of the factors had significant effects on the parameters, a post hoc procedure using Scheffe's F-test was used for multiple comparisons between groups. For the statistical analyses, we used a computer program (SPSS for Windows, Version 12.0) that was capable of performing factorial ANOVA with unequal replication, since the number of replicates for each group was two or three in the storage experiment (Table 1).

3. Results

3.1. Storage of epididymides at refrigerated temperatures

First, we examined the period for which spermatozoa maintained fertilization ability when stored within epididymides at refrigerated temperatures. Epididymides were collected from B6 or BDF1 males and stored at 5 °C or 7 °C for 0–4 d. There were effects of strain and storage period on fertilization rates ($P < 0.05$; Table 1) but no significant interactions. The BDF1 spermatozoa retained superior fertilizing ability than B6 spermatozoa at every time-point and at both temperatures tested, except in the case of 3-d storage at 7 °C. We anticipated that spermatozoa retrieved from epididymides stored for longer than 3 d at 7 °C would achieve >50% fertilization rates for both strains (Table 1).

3.2. Transportation of epididymides at refrigerated temperatures

The temperatures during transport ranged from 4.0 to 5.5 °C and 6.0 to 8.0 °C for the pre-set temperatures of 5 and 7 °C, respectively. Both strain and temperature affected fertilization rates subsequent to epididymal transportation ($P < 0.01$ and $P < 0.05$, respectively; Table 2). After transportation and freeze/thaw cycles, only strain affected fertilization rates ($P < 0.01$; Table 2). There was no significant interaction. As in the first series of experiments, BDF1 spermatozoa maintained high fertilizing capacities (76.3 and 77.5%), irrespective of the transportation and storage temperatures. These spermatozoa yielded acceptable fertilization rates (59.1 and 66.4%, respectively), even after freezing and thawing. In contrast, superior fertilization rates were achieved with B6 spermatozoa that were transported at 7 °C than for those transported at 5 °C ($P < 0.05$). Similar trends

Table 2
Rates of fertilization using mouse spermatozoa retrieved after epididymal transport, stored at low temperature and then cryopreserved

Strain	Transport temperature (°C)	After transportation	After transportation and sperm cryopreservation
B6D2F1	5	427/551 (77.5)	256/433 (59.1)
	7	335/439 (76.3)	336/506 (66.4)
B6	5	209/503 (41.6)[a]	97/351 (27.6)
	7	297/437 (68.0)[a']	136/326 (41.7)

Results from three replicate experiments; [a,a'] $P < 0.05$. Values in parentheses are in percent (%).

were observed for the success of IVF using frozen-thawed spermatozoa, although the differences were not significant ($P > 0.05$).

For proportions of fresh spermatozoa with motility and progressive movement after pre-incubation. There were effects of strain on the motility rate (78–89% for BDF1 and 62–85% for B6; $P < 0.05$) and progressive motility rate (32–55% for BDF1 and 25–42% for B6; $P < 0.05$). Temperature had no effect on either parameter ($P > 0.05$). There was an interaction between strain and temperature for the motility rate ($P < 0.05$) and correlation between fertilization rates and progressive movements of spermatozoa ($r = 0.61$, $P < 0.05$).

3.3. Embryo culture and transfer

To assess the normality of embryos produced by IVF using transported spermatozoa, embryos were cultured up to the blastocyst stage for 96 h. Development into blastocysts occurred in 92.0% (5 °C) and 93.7% (7 °C) of the BDF1 fertilized embryos and 69.4% (5 °C) and 74.1% (7 °C) of the B6 fertilized embryos. These rates were not different ($P > 0.05$) from those obtained in our conventional IVF and embryo culture program using fresh spermatozoa (94.4% for BDF1 and 77.1% for B6). The two-cell embryos from the B6-7 °C group were transferred into the oviducts of recipient ICR females. The recipients ($n = 7$) became pregnant and gave birth to 41 normal offspring. The implantation and birth rates were 76.2% (64/84) and 48.8% (41/84), respectively.

4. Discussion

We were interested in the feasibility of transporting non-frozen mouse spermatozoa between facilities, which would provide several advantages over transporting cryopreserved spermatozoa. First, the potential for damage to spermatozoa as a result of freezing and thawing could be avoided. The fertilizing ability of cryopreserved mouse spermatozoa (especially those from B6-derived strains) is frequently decreased after thawing, unless both the sender and recipient are highly skilled in freezing and thawing techniques [17,18]. Second, no special containers or conditions are needed to transport non-frozen epididymides, whereas the safe transport of cryopreserved spermatozoa requires large liquid nitrogen containers (typically dry-shippers), which are usually returned to the

sending facility after delivery, making the transportation of cryopreserved spermatozoa both costly and inconvenient. Based on the present study, these obstacles can be circumvented by transporting fresh epididymides under ordinary refrigerated conditions.

In this study, fresh epididymides were transported by a domestic courier service that guaranteed transportation under refrigerated conditions. Temperature fluctuations in the container during transportation were minimal (~1 °C). Improved packaging methods may maintain optimal temperatures for longer periods, thereby allowing transport in cases where constant refrigeration may not be possible, such as overseas transport. The ability of epididymal spermatozoa, particularly B6 spermatozoa, to tolerate temperature fluctuations during transportation should also be examined. Even in cases of delayed transportation or temperature fluctuations, intracytoplasmic sperm injection [7] or partial zona dissection [8] of oocytes would probably facilitate fertilization, as has been reported for spermatozoa retrieved 7 d after the death of a donor male mouse [15].

Recently, protocols for non-frozen storage of isolated spermatozoa have been optimized [13]. F1 hybrid (B6C3HF1) spermatozoa maintained motility in media for 1 d at 22 °C, whereas B6 and C3H/HeN spermatozoa lost motility very rapidly. In the present study, BDF1 spermatozoa had better storage potential and even B6 spermatozoa retain good motility and fertilizing ability within the epididymides for at least 3 d. Since spermatozoa generally have the ability to move for only a limited time period in the female genital tract, it is reasonable to store them in a quiescent state. Furthermore, the epididymis secretes factors that protect epididymal spermatozoa from premature acrosome reactions [19]; these factors probably stabilize sperm membrane structures, including the nucleus and promote cell integrity. Interestingly, it has been reported that fertilization rates decreased to less than 50% after storage for 2 d in the epididymides, although progressively motile spermatozoa could be retrieved from epididymides stored at 5 °C for 8 d [9]. The sperm plasma and acrosomal membranes, which should remain intact before capacitation, may suffer more damage than the components required for movement, such as those of the midpiece and tail. Sperm nuclei seem to be much more stable, since normal live fetuses can be obtained by microinsemination using spermatozoa retrieved from the epididymides 20 d after death [20].

Many valuable transgenic and knockout strains have been produced using mice with the B6 genetic background, and we believe that sperm transportation is the best way to exchange these valuable genetic resources. Therefore, it is worth emphasizing that B6 spermatozoa transported within epididymides retain acceptable fertility for at least 3 d at 7 °C. We also have confirmed that the B6 embryos, thus, produced can develop into full-term offspring. Thus, it may be possible for laboratories that do not specialize in sperm cryopreservation to receive fresh epididymides of strains of interest and to raise live mice. Furthermore, we have found that B6 spermatozoa transported within the epididymides can be cryopreserved safely for later use. We have found, and others have reported, practical fertilization rates of 40–61% for B6 and its transgenic derivative mouse lines through the selective use of high motility spermatozoa after freezing and thawing [17,18]. The combination of epididymal transport and subsequent sperm cryopreservation may facilitate the mouse resource banking programs currently in worldwide operation, including our RIKEN Bioresource Center.

In conclusion, mouse spermatozoa can be transported under non-freezing conditions by storing them within epididymides. This method appears to be an easier and safer alternative

to shipping cryopreserved spermatozoa, and should facilitate the distribution between research facilities of mouse strains, especially genetically engineered mice with the B6 genetic background.

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LETTER

Birth of Mice Produced by Germ Cell Nuclear Transfer

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Summary: That mammals can be cloned by nuclear transfer indicates that it is possible to reprogram the somatic cell genome to support full development. However, the developmental plasticity of germ cells is difficult to assess because genomic imprinting, which is essential for normal fetal development, is being reset at this stage. The anomalous influence of imprinting is corroborated by the poor development of mouse clones produced from primordial germ cells (PGCs) during imprinting erasure at embryonic day 11.5 or later. However, this can also be interpreted to mean that, unlike somatic cells, the genome of differentiated germ cells cannot be fully reprogrammed. We used younger PGCs (day 10.5) and eventually obtained four full-term fetuses. DNA methylation analyses showed that only embryos exhibiting normal imprinting developed to term. Thus, germ cell differentiation is not an insurmountable barrier to cloning, and imprinting status is more important than the origin of the nucleus donor cell per se as a determinant of developmental plasticity following nuclear transfer. genesis 41:81–86, 2005. © 2005 Wiley-Liss, Inc.

Key words: nuclear transfer; cloning; mouse; primordial germ cell; genomic imprinting

Since the birth of the sheep Dolly, the first eutherian mammal cloned from an adult cell, successful cloning using somatic cell nuclear transfer has been reported for the mouse, bovine, goat, pig, cat, rabbit, mule, horse, and rat (for review, see Tamada and Kikyo, 2004). Although the efficiency of cloning is low, it appears that at least some somatic cell genomes can acquire totipotency following transfer into enucleated oocytes. Somatic cell cloning is now being applied extensively to the production of clones of individuals, and to the generation of animals with genetic modifications for agricultural and pharmaceutical purposes. However, little is known about the developmental plasticity of the genome of the germ cell lineage. Only midgestation fetuses have been obtained from mouse primordial germ cells (PGCs), leading some to invoke "hemipotency" of the genome in the germ cell lineage (Kato *et al.*, 1999; Lee *et al.*, 2002; Yamazaki *et al.*, 2003). This limited efficiency of development of embryos cloned from PGCs may be attributed to the low plasticity of the germ cell genome attained during germ cell differentiation, or to erasure of the genomic imprinting "memory," known to occur in PGCs at embryo gestational day 11.5 in the mouse (Lee *et al.*, 2002).

Genomic imprinting in eutherian mammals is an epigenetic mechanism that ensures parent-allele-specific expression in some genes (imprinted genes) and plays essential roles in development and adult behavior (Reik and Walter, 2001; Li *et al.*, 1999). Imprinting "memories" are erased during early germ cell development and then reestablished in a parent-specific manner, depending on the sex of the individual. We previously examined the expression pattern of imprinted genes in fetuses cloned from fetal PGCs at embryo gestational days 11.5–13.5 (assuming the morning of the postcopulatory plug as day 0.5), and found that loss of their monoallelic expression proceeded stepwise from day 11.5 and was completed by day 12.5 (Lee *et al.*, 2002). Fetuses cloned from PGCs at days 12.5–13.5 arrested their development around day 8.5 in recipient foster mothers, as has been reported for PGCs at days 14.5–16.5 (Kato *et al.*, 1999). Those from nuclei of PGCs at day 11.5 developed furthest (to day 11.5), although they exhibited marked variation in their developmental potential, presumably reflecting the dynamic process of imprinting erasure in donor nuclei (Lee *et al.*, 2002). To extend this work, we created clones using PGCs from embryos at days 10.5 and 11.5 and examined whether such differentiated germ cell genomes could be fully reprogrammed by nuclear transfer. To ensure the accurate identification of PGCs, we used transgenic mice expressing green fluorescent protein (GFP) driven by the PGC-restricted *Oct-4* promoter (Yeom *et al.*,

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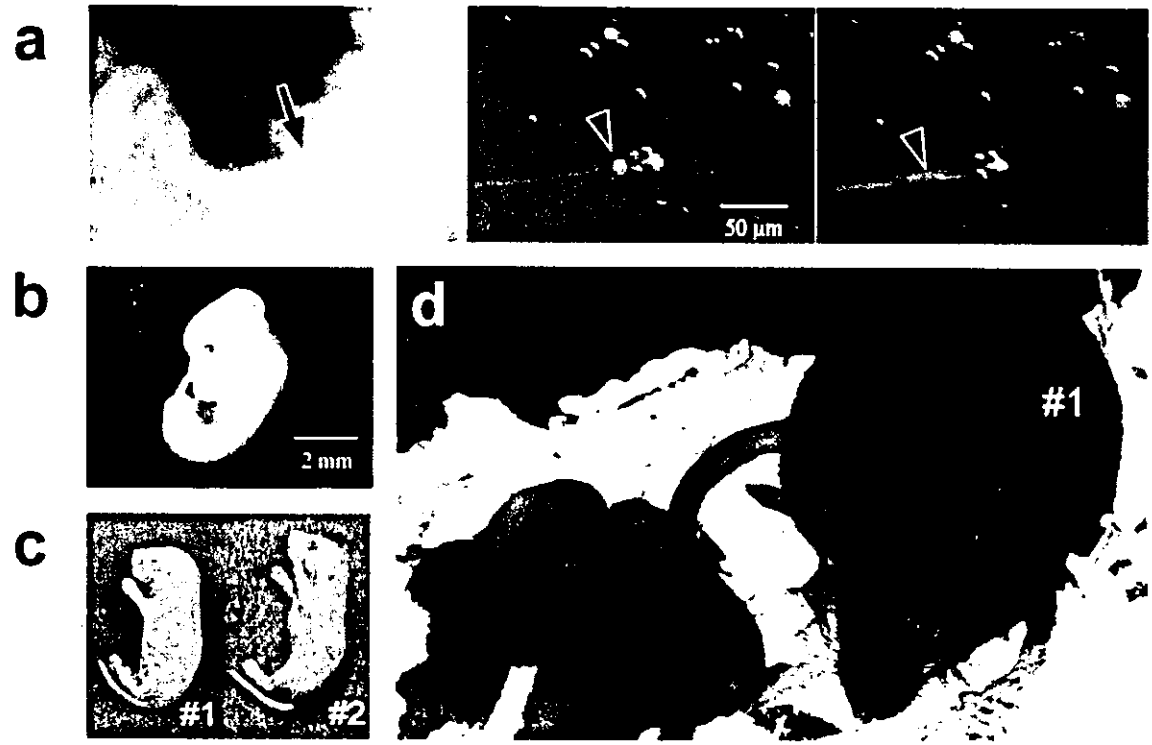

FIG. 1. Nuclear transfer with PGCs. **a:** PGCs reaching the genital ridges (arrow) at day 10.5 (left), and cells retrieved from the genital ridge at gestational day 10.5 (middle and right). PGCs were identified by their fluorescent markers and selected for nuclear transfer into enucleated oocytes (arrowheads). **b:** A gestational day 12.5 fetus cloned from a day 11.5 PGC. It was alive and appeared normal. As far as we examined, day 12.5 was the most advanced stage to which day 11.5 PGC clones could develop normally. **c:** Live female pups (#1 and #2) produced from day 10.5 PGCs by nuclear transfer. **d:** Both pups grew into normal adults and gave birth to offspring with normal litter sizes.

1996; Yoshimizu *et al.*, 1999) (Fig. 1a). We confirmed that all the GFP-expressing cells were positive for PGC7/Stella and alkaline phosphatase (data not shown), both of which are markers for mouse PGCs (Yoshimizu *et al.*, 1999; Saitou *et al.*, 2002; Sato *et al.*, 2002).

Approximately 60–80% of enucleated oocytes survived injection with PGC nuclei. When embryos were reconstructed with PGC nuclei from day 11.5 embryos (presumably in the G1 stage of the cell cycle), more than half of them cleaved within 24 h, and of these 63% reached the four-cell stage within the next 24 h (Table 1). These rates of development in vitro are similar to those reported previously (Lee *et al.*, 2002). Approximately 50% of the cloned embryos implanted following transfer into the oviducts of pseudopregnant females (Table 1). Live fetuses were retrieved at days 9.5, 10.5, 11.5, and 12.5, but not at days 13.5, 14.5, or 15.5 (Table 2). The three fetuses retrieved at days 11.5 and 12.5 appeared normal and had beating hearts (Fig. 1b). Thus, we concluded that day 12.5 was the most advanced stage of normal development that could be attained by embryos cloned from day 11.5 PGCs.

Based on these results, we evaluated the developmental potential of PGC nuclei from day 10.5 embryos. Cloned embryos reconstructed from these nuclei developed in vitro at a similar efficiency as those cloned from day 11.5 PGCs (Table 1). We reasoned that the relatively low cleavage rates of these PGC clones compared with those from cumulus cell or immature Sertoli cell nuclei (Ogura *et al.*, 2000a) could be attributable to rapid proliferation of the donor PGCs, which may cause cell cycle asynchrony between the recipient oocyte and the PGC nucleus. Embryos cloned from embryonic stem (ES) cells, which are also rapidly dividing cells, have shown a similar developmental pattern (Wakayama *et al.*, 1999). To test this possibility, we synchronized day 10.5 PGCs at metaphase by treatment with nocodazole and used the resulting cells as nuclear transfer donors. We found that the cleavage rate was indeed significantly improved ($P < 0.005$, Table 1). This strongly suggests that asynchrony of the cell cycle, not poor developmental plasticity of the PGCs, was the major cause of the one-cell arrest of these embryos cloned from PGCs. When 563 embryos reconstructed from day 10.5 PGCs were transferred into recipient females, 15 (2.7%) generated retrievable embryonic products at term (day 19.5). Of these, four contained fetuses (two alive and two stillborn), with the remaining 11 containing only a well-developed placenta with no associated fetus. The live-born PGC clones were both females that developed into normal adults of proven fertility (Fig. 1d).

Table 1
Development of Embryos Derived From Enucleated Oocytes Injected With Primordial Germ Cell (PGC) Nuclei

Day of PGC	Cell cycle	Method of nuclear transfer	No. cultured*	Cleaved (%)	4-cell (% of cleaved)	Transferred	Implanted (%)	Retrievable conceptuses at term (%)	Offspring (%)
11.5	Presumptive G1	Injection	1,512	831 (55.0)	543 (65.3)	521	256 (49.1)	—	—
10.5	Presumptive G1	Injection	2,018	1,011 (50.1)[a]	611 (60.4)	441	252 (57.1)	13 (2.9)	4 (0.9)
10.5	M	Fusion	269	189 (70.3)[b]	133 (70.4)	122	60 (49.2)	2 (1.6)	0 (0.0)
				(Day 10.5 PGC total)		962	508 (52.8)	15 (1.6)	4 (0.4)
Immature Sertoli cell	Presumptive G1	Injection	503	335 (66.6)	311 (92.8)	288	160 (55.6)	37 (12.8)	28 (9.7)

*Oocytes successfully injected (G1) or fused (M).
[a,b] $P < 0.005$ (one-way ANOVA).

Table 2
Number of Live Fetuses Retrieved Following Transfer of Embryos From Day 11.5 PGCs

Day of gestation	No. live fetuses/ those implanted
9.5	6/173
10.5	6/62
11.5	2/30
12.5	1/47
13.5	0/14
14.5	0/82
15.5	0/113

We analyzed the DNA methylation status of differentially methylated regions of imprinted genes in day 11.5 PGC clones surviving to midgestation and the day 10.5 PGC clones that reached term. All placental and tail-tip tissues examined contained both a methylated and an unmethylated allele for *Peg5/Nnat*, *Peg3*, and *H19*, each indistinguishable from its respective normal methylation imprint (Fig. 2). The timing of methylation erasure relative to other imprinted genes is early for *Peg5/Nnat* and *Peg3*, and is intermediate for *H19* (Lee *et al.*, 2002). Moreover, the imprinting status of clones may reflect that of their respective nucleus donors (Inoue *et al.*, 2002). This suggests that normal development was restricted to those PGC donor nuclei that retained parental imprinted memory for most, if not all, imprinted genes. The difference in developmental ability of clones derived from PGC nuclei at days 10.5 and 11.5 thus probably reflects a subpopulation of PGCs that had not initiated erasure of their imprinting memory. This assumption is consistent with results from analysis of the DNA methylation status of imprinted genes in donor PGCs at days 10.5–12.5. These cells are heterologous populations in terms of their DNA methylation status, as the former group has more nuclei exhibiting monoallelic methylation marks that have not yet been erased (Lee *et al.*, 2002). However, a recent study showing the full-term development of parthenogenetically reconstructed embryos indicates that the control of imprinted gene expression levels is more complex than we had thought (Kono *et al.*, 2004). The expression levels of certain genes (*H19* and *Igf2*, and probably *Gtl2* and *Dlk1*) apparently affected the expression profiles of a broad range of other imprinted genes, and thus determined the developmental potential of embryos. It will be interesting to see whether the same is true for the development of clones from PGC. A wide-range analysis using cloned fetuses from midgestation PGCs would settle this question.

All term placentas of conceptuses cloned from day 10.5 PGCs, including the placenta-only ones, showed hypertrophic development, characteristic of somatic cell clones in the mouse (Fig. 3) (Wakayama and Yanagimachi, 1999a). This placental hypertrophy, together with the frequent postimplantation loss of such embryos (Wakayama and Yanagimachi, 1999b), indicates that the genomes of day 10.5 PGCs still retain a somatic cell genome type in

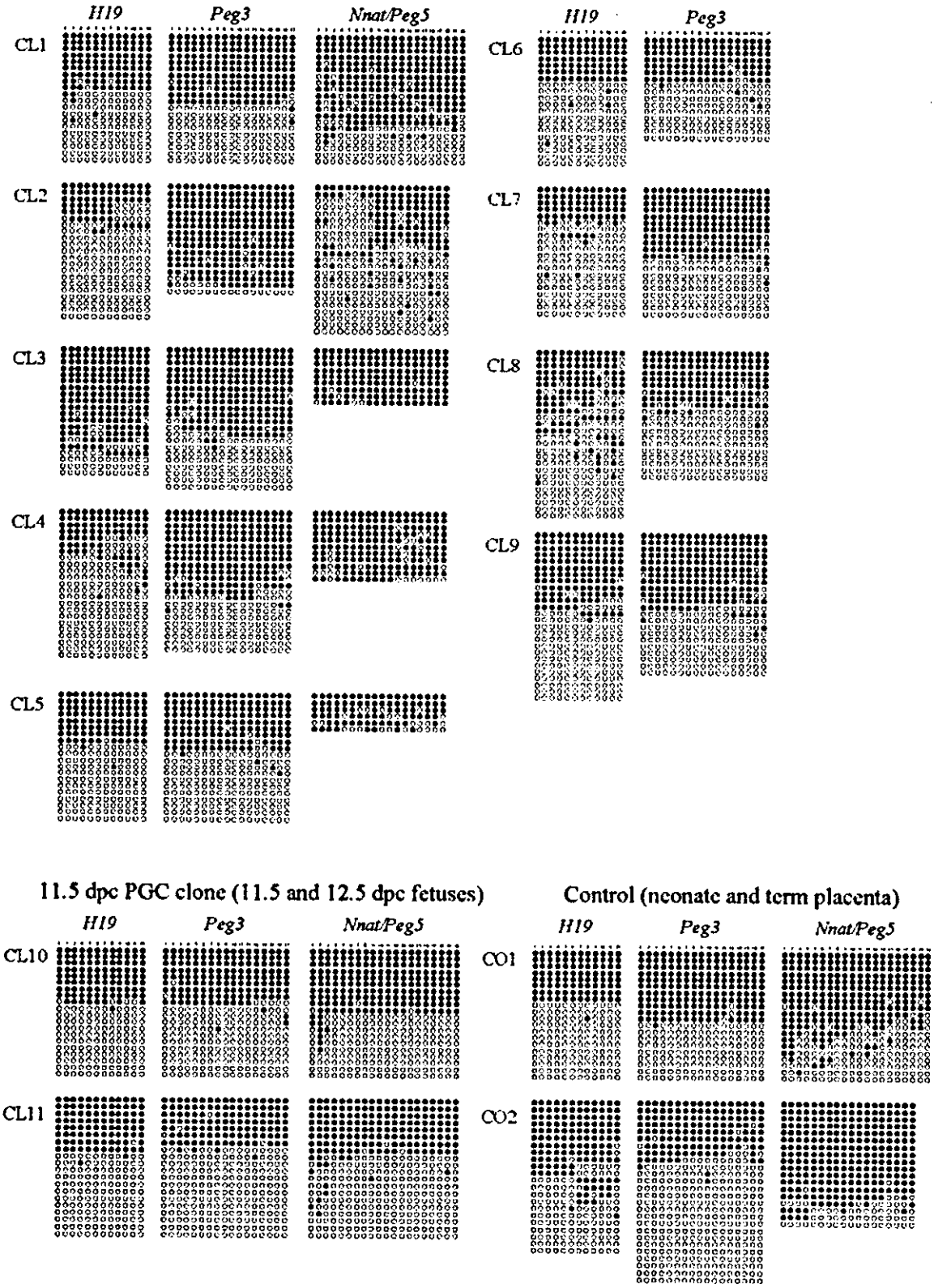
10.5 dpc PGC clone (neonate and term placenta)
H19
Peg3
Nnat/Peg5
CL1
CL2
CL3
CL4
CL5
H19
Peg3
CL6
CL7
CL8
CL9
11.5 dpc PGC clone (11.5 and 12.5 dpc fetuses)
H19
Peg3
Nnat/Peg5
CL10
CL11
Control (neonate and term placenta)
H19
Peg3
Nnat/Peg5
CO1
CO2

FIG. 2

terms of their reprogrammability in the egg cytoplasm. It is reasonable to assume that the female (oocyte) and male (sperm) germ cell genomes undergo some epigenetic modifications during their early development, so that they can be correctly reprogrammed at fertilization to participate in the formation of a totipotent zygotic genome (Fulka *et al.*, 2004). Somatic cell genomes presumably bypass this process and therefore are erroneously reprogrammed when transferred into the egg cytoplasm (Dean *et al.*, 2001). Our results indicate that this (unknown) epigenetic modification process—if present—must exist at day 11.5 or later in the mouse.

Differentiation of the germ cells equips them uniquely to transmit their genome to the next generation. We have shown here that PGCs recovered as late as gestational day 10.5 are able to support full development following nuclear transfer. This suggests that the germ cell genome can be fully reprogrammed even after the PGCs have undergone distinctive differentiation (Saitou *et al.*, 2002; McLaren, 2003) and have reached the genital ridge (typically around day 10.5 in the mouse). These findings support the hypothesis that, in general, imprinting status is more important than the origin of the nucleus donor cell per se as a determinant of definitive developmental plasticity following nuclear transfer.

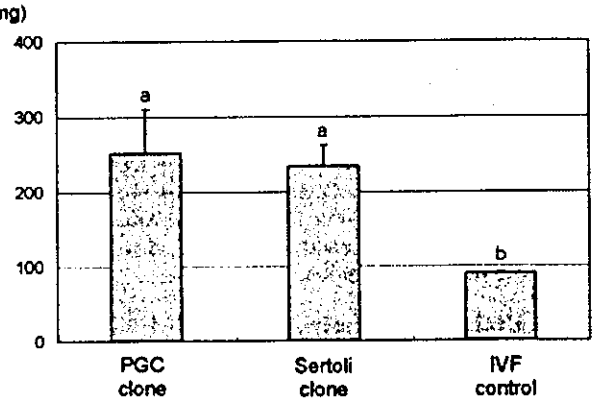

FIG. 3. Placental weights of clones and in vitro fertilization (IVF) controls at term. The mean weights of placentas cloned from day 10.5 PGCs (n = 15) and from immature Sertoli cells (n = 11) were significantly larger than those of genotype-matched IVF controls (n = 14). There was no significant difference in mean placental weight between PGC and Sertoli cell clones. $^{a,b}P < 0.05$ (by Scheffe's F-test). Error bar = SEM.

MATERIALS AND METHODS

Preparation of Donor Cells

For preparation of donor PGCs, B6D2F1 females were mated with *Oct-4*/GFP transgenic males (GOF-18/delta PE/GFP, 129/Sv-*ter* background), and sacrificed at days 10.5 or 11.5 of embryonic development. The PGC-specificity of GFP fluorescence was endowed by the *Oct-4* fragment transgene in the genome of strain GOF-18/delta PE (Yeom *et al.*, 1996; Yoshimizu *et al.*, 1999). Genital ridges were removed from fetuses and treated with 0.1 mg collagenase (Sigma, St. Louis, MO) in Complete Blastocyst Medium (CBM) (Irvine Scientific, Santa Ana, CA) at 37°C for 40 min to suspend single cells. The media used for culturing PGCs were kept serum-free throughout to avoid alteration of the genomic imprinting status of PGCs (Khosla *et al.*, 2001).

Nuclear Transfer

Nuclear transfer was carried out as described (Wakayama *et al.*, 1998; Ogura *et al.*, 2000b; Inoue *et al.*, 2003). The oocytes were collected from 8-week-old B6D2F1 females superovulated by injection with PMSG (7.5 IU) and hCG (7.5 IU) at 48-h intervals. At 15 h after the hCG injection, cumulus-oocyte complexes were collected from the oviducts and the cumulus cells were allowed to disperse in KSOM medium (Lawitts *et al.*, 1993) that contained 0.1% bovine testicular hyaluronidase (Sigma). The oocytes were enucleated in Hepes-buffered KSOM medium containing 7.5 μg/mL cytochalasin B (Calbiochem, San Diego, CA). The nuclei from PGCs were transferred into enucleated oocytes using intracytoplasmic injection with piezo-driven micromanipulators (Primetech, Ibaraki, Japan: Wakayama *et al.*, 1998; Inoue *et al.*, 2003), or by electrofusion (Ogura *et al.*, 2000b). In initial experiments, we selected relatively small PGCs (~15 μm in diameter) for nuclear transfer, in attempts to select relatively homogeneous populations that would be compatible with recipient oocytes. In later experiments, PGCs were arrested at metaphase by exposure to 0.4 μg nocodazole (Sigma) in CBM for 4 h and subsequently used for nuclear transfer by electrofusion. Unlike experiments on nuclear transfer using nontreated PGCs, following the transfer of presumptively post-S-phase nocodazole-treated PGCs the polar body containing its donor sister chromosomes was extruded to restore diploidy. Oocytes successfully injected or fused with donor PGCs were cultured in

FIG. 2. Bisulfite genomic sequence analysis of PGC clones. Fetuses, term placentas, and neonates were analyzed for *H19*, *Peg3*, and *Nnat/Peg5* in day 10.5 PGC clones, day 11.5 PGC clones, and controls. Open circles represent unmethylated CpG sequences, and filled circles indicate methylated CpGs. All of the placental and tail-tip tissues examined contained methylated and unmethylated alleles for *Peg5/Nnat*, *Peg3*, and *H19* that were indistinguishable from their respective normal methylation imprints, except for hypermethylated *Peg3* alleles in the second clone. For some term placentas, the *Nnat/Peg5* sequences were not analyzed because this gene showed tissue-specific hypermethylation in placentas, including the control (CO2). CL1, first live day 10.5 PGC clone neonate (tail-tip); CL2, second live day 10.5 PGC clone neonate (tail-tip); CL3, stillborn PGC clone (placenta); CL4-9, placenta-only conceptus at term; CL10, a gestational day 11.5 fetus cloned from a day 11.5 PGC; CL11, a gestational day 12.5 fetus cloned from a day 11.5 PGC; CO1, control IVF neonate (tail-tip); CO2, control IVF term placenta.

KSOM medium for 48 h and four-cell embryos were transferred into the oviducts of day 0.5 ICR strain pseudopregnant females, which had been mated with vasectomized males. The recipient females were examined for the presence of conceptuses at midgestation or term by cesarean section. Retrieved term fetuses were nursed by lactating ICR females. Control embryos were produced by nuclear transfer using immature Sertoli cells (Ogura *et al.*, 2000a) or by conventional in vitro fertilization (IVF) (Toyoda *et al.*, 1989).

DNA Methylation Analysis

Genomic DNA was prepared from fetuses, placentas, and tail-tip tissues of PGC clones and genotype-matched controls, using ISOGEN (Nippon Gene, Japan). Purified DNA was treated with a sodium bisulfite solution as described previously (Raizis *et al.*, 1995). The *H19* promoter region, the 5′ upstream region of *Peg3*, and the 5′ upstream region of *Peg5* were amplified using *ExTaq* DNA polymerase (TaKaRa, Japan) for 30 cycles under the following conditions: 96°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The PCR primers used were as follows: H19 bi F: 5′-GGA ATA TTT GTG TTT TTG GAG GG-3′; H19 bi R: 5′-TTA AAC CCC AAC CTC TAC TTT TAT AAC-3′; Peg3-CT-IF: 5′-TTT TGT AGA GGA TTT TGA TAA GGA GG-3′; Peg3-CT-IR2: 5′-CCC CAA ACA CCA TCT AAA CTC TAC-3′; Peg5 bi F: 5′-GAG GAT ATA AGT TTT ATT TTG AAA TTA GAA G-3′; Peg5 bi R: 5′-TAC CTT AAA TAC CCT CTT ACC ACC TAA A-3′.

Amplified fragments were cloned into the plasmid vector pGEM-T Easy (Promega, Madison, WI) and sequenced using BigDye Terminator Cycle Sequencing Kit (v. 3.1) using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Statistical Analysis

The proportions of embryos that reached two cells, four cells, or term were transformed using arcsine transformation and analyzed by repeated-measure one-way ANOVA. The weights of placentas from clones and controls were analyzed by repeated-measure one-way ANOVA, followed by a multiple comparison test (Scheffe's F-test). $P < 0.05$ was assumed statistically significant.

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Establishment of an Efficient BAC Transgenesis Protocol and its Application to Functional Characterization of the Mouse Brachyury Locus

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Abstract: *Transgenesis using large DNA such as YAC or BAC has extended the range of applications in functional genomics. Here we describe an efficient BAC transgenesis protocol using a simple BAC DNA preparation method adopted from YAC DNA purification methods. This method allowed us to isolate BAC DNA from small scale culture of BAC-containing cells in sufficient quantity and purity for microinjection. More than 40 founders have been produced with linearized BAC DNA prepared by this method, and 85% of them contained intact BAC transgenes. In contrast, when circular BAC DNA was injected, an approximately three-fold reduction of transgene integration rate was observed and fewer intact transgene integrations were obtained. A line of transgenic mice carrying a 170-kb BAC clone generated in this way successfully rescued tail and embryonic lethality phenotypes of the mouse* Brachyury (T) *mutants, further demonstrating the utility of this method in functional analysis of the mouse genome.*

Key words: *BAC, development, T-locus, transgenesis*

Introduction

Transgenic technology has been extremely useful and successful for studying functions of genes of interest at an organism level. However, there are several technical limitations in conventional transgenic techniques. Since introns and essential regulatory elements required for correct *in vivo* expression tend to be omitted in the constructs, transgene expression often fails to follow the expression patterns of the corresponding endogenous gene. The chromosome 'position-effect' affects foreign gene expression, depending on the chromosomal integration site, compromising the transgene expression. In addition, the maximum size of the transgene is only

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40–50 kb, due to constraints on the insert length that can be cloned in phage or cosmid vectors. These limitations can be overcome by using cloning systems which accommodate submegabase DNA such as YAC (yeast artificial chromosome) or BAC (bacterial artificial chromosome) in the generation of transgenic mice (reviewed by Giraldo and Montoliu [7]). The ability to introduce large size DNA into mouse germ lines should help in attempts to isolate responsible genes for existing mutations, or induced mutations generated through the efforts of several 'large scale' mutagenesis projects operating in international mouse communities [11].

Although BAC DNA can be easily isolated by conventional plasmid preparation protocol for analytical work, care must be taken in preparing intact large DNA inserts for microinjection [5, 22, 24]. Takahashi *et al.* [22] compared three purification procedures for BAC DNA, and found that transgenic animals with intact BAC inserts were obtained only with BAC isolated by CsCl gradient ultra-centrifugation followed by linearization with restriction enzyme, pulsed field gel electrophoresis (PFGE) separation, and β-agarase digestion. We devised a similar but simpler procedure for BAC DNA isolation adopted from a YAC DNA purification method [17], and have generated 43 founder transgenic mice. Twenty nine mice in thirty four informative cases (85.3%) seemed to have intact BAC transgenes. Genes contained in the BAC transgene appeared to be expressed in a correct fashion [8] and functional.

Mouse *Brachyury* (*T*) mutation is a semidominant mutation causing a short tail phenotype when heterozygous (*T*/+), or embryonic lethality when homozygous. Another mutation, *tct*, closely linked to the *T* gene interacts with *T* to cause a tailless phenotype in the double heterozygote, *T*/*tct* [3, 6]. The *T* gene was identified by Herrmann *et al.* [9], but the molecular nature of the *tct* mutation remains to be elucidated [1, 12, 14, 15, 16]. Here, we introduced a 170-kb BAC containing the entire *T* gene, and completely rescued the tail phenotypes of *T*/+ and of *T*/*tct*. The embryonic lethal phenotype associated with the *T*-locus was also completely corrected by the presence of the BAC transgene.

Materials and Methods

BAC clones: BAC clones used in this study were isolated from a mouse BAC library, CITBCJ7, supplied by Research Genetics (Cat.# 96050, Huntsville, AL, USA). Mouse genomic DNA is cloned into pBelloBAC11 vector [19] in this library. Details of BAC42, 132, and 213 clones were described by Kokubu *et al.* [13].

Purification of BAC DNA for microinjection:

Preparation of cell plugs: Bacterial cells containing BAC clone were cultured overnight in 10 ml of LB with 12.5 μg/ml of chloramphenicol, collected by centrifugation at 5,000 rpm for 15 min, and resuspended in 10 ml of PIV buffer (1 M NaCl, 10 mM Tris-Cl, pH 7.6). The suspension was centrifuged again, resuspended in 1.6 ml of the PIV buffer, and warmed to 37°C. The warmed suspension was mixed thoroughly with an equal volume of molten agarose, 1.6% InCert (FMC BioProducts, Rockland, ME), previously melted and warmed at 50°C. One hundred microliter of the mixture was pipetted into the well of the agarose plug mold (LKB/Amersham Pharmacia, Piscataway, NJ), and the mold was stood at 4°C to allow the agarose to solidify.

Lysozyme/RNase digestion: The agarose plug containing bacterial cells was placed in a 50 ml plastic tube, and 1 ml per plug of EC lysis solution, 6 mM Tris-Cl, pH 7.6, 1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, and 0.5% Sarkosyl were added. The mixture was shaken gently at room temperature for 15 min, then the EC lysis solution was replaced with fresh EC lysis solution containing 1 mg/ml lysozyme and 20 μg/ml RNase, and incubated at 37°C overnight.

Proteinase K digestion: The EC lysis solution was aspirated off and 1 ml per plug of ESP buffer, 0.5 M EDTA, pH 9.0, and 1% Sarkosyl were added. The mixture was shaken gently at room temperature for 15 min, then the buffer was replaced with 1.5 ml per plug of ESP containing 50 μg/ ml of proteinase K. The plugs were incubated at 50°C overnight with shaking. After proteinase K digestion, the plugs were washed twice with TE, 10 mM Tris-Cl, pH 7.5 and 1 mM EDTA, for 30 min each at room temperature, and stored at 4°C until use.

Linearization of BAC clone:

A: λ-Terminase treatment. Agarose plugs containing BAC DNA were washed in TE for 30 min twice,

and washed three times in 10 mM Tris-Cl, pH7.5. The plugs were soaked in 1 × Terminase buffer without Terminase, and kept on ice for 1 h. The composition of 5 × λ-Terminase buffer was 65 mM Tris-Cl, pH 8.0, 15 mM $MgCl_2$, 375 mM KCl, 0.05% Triton X-100, 5 mM ATP, 2.5 mM EDTA, 30 mM β-mercaptoethanol, and 25 mM spermidine. One agarose plug was placed in a tube containing 100 μl of 1 × Terminase buffer, 0.5 mM ATP, and 8 units of λ-Terminase (TAKARA Shuzo, Tokyo, Japan), and the tube was kept on ice for 1 to 2 h, then incubated at 30°C for 6 h to 16 h.

B: Use of rare cutting restriction enzyme. Uncut BAC clone was separated from the *E. coli* chromosome on PFGE: 0.5 X TBE, 1% low melting point (LMP) agarose, SeaPlaque GTG (FMC BioProducts, Rockland, ME). An agarose fragment containing BAC was excised, and washed twice in 1 × buffer appropriate for restriction digestion for 30 min. The agarose block was soaked in restriction enzyme solution (60 units/ml) in a test tube, incubated on ice for 1 h, and transferred to a 37°C incubator for overnight incubation. The agarose plug was inserted into a slot of another pulsed field gel, sealed with 1% LMP agarose and subjected to PFGE.

Pulsed-Field Gel separation of BAC insert: Agarose plugs containing linearized BAC DNA were loaded onto a preparative pulsed field gel of 0.5 X TBE, and 1% SeaPlaque GTG agarose. Pulsed field gel electrophoresis was performed under conditions optimized to separate the BAC from the *E. coli* genome. After the PFGE run, marker lanes on either side of the preparative lane as well as about 5 mm of the preparative lane were cut off and stained with ethidium bromide. The position of the BAC band was marked with a sterile razor blade, and the part of the preparative lane containing the BAC DNA was excised. The excised gel slice was twice equilibrated in at least 10 volumes of the YAC buffer (10 mM Tris-Cl, pH 8.0, 0.1 mM EDTA, 100 mM NaCl, 30 μM spermine, 70 μM spermidine) for 30 min, then at 4°C for 16 h. The gel slice was placed into a 2.0 ml Eppendorf tube, and the remaining buffer was removed with a micropipette. The agarose was melted at 68–70°C for 15 min or longer. The agarose should completely melt into the solution at this step. The tube was placed in a 42°C water bath and incubated for 15 min. An equal volume of β-agarase solution, 3% sucrose in the YAC buffer containing β-agarase (NEB, Beverly, MA), prewarmed at 42°C was added into the agarose solution. Four units of β-agarase should be added per 100 μl of molten agarose. After incubation at 40°C for ~3 h, the tube was spun at 5,000 rpm for 5 min at room temperature. The supernatant was transferred to a Microcon-100 filtration device (Amicon, Danvers, MA) with a wide bore pipette tip, spun at 4,000 rpm for 5 min and washed twice with YAC buffer. The DNA solution in the upper chamber of the Microcon-100 was collected, and this solution was directly used for microinjection. To determine concentration and to check the integrity of the BAC DNA, a small portion of the DNA solution was electrophoresed on a pulsed field gel and compared with known amounts of standard DNA, e.g. λ DNA.

To isolate circular BAC DNA for microinjection, BAC DNA was obtained from a 500 ml culture of BAC-containing bacteria using a QIAGEN Large Construct Isolation kit (QIAGEN, K.K., Tokyo, Japan) according to supplier's instructions.

Injection into mouse fertilized eggs: BAC transgene was microinjected into the male pronuclei of fertilized eggs obtained from C57BL/6 J mating [20]. A total of two to three hundreds eggs were regularly used for injection of each BAC DNA construct. Transgenic mice were screened using genomic DNAs collected from tail biopsy.

Genotyping by PCR: To detect BAC transgene integration, Southern blot analysis or PCR typing is generally performed. When BAC transgene is linearized by λ Terminase at the cos site, the BAC transgene has small portions of BAC vector sequences at both ends of the insert. Primers used for amplification of the left end (T7 promoter seq. side) of the pBeloBAC11 vector were pBelo3; 5'-CAATGGAAGTCCGAGCTC and BAC2; 5'-GTCGACTCTAGAGGATC (product size, 450 bp), and primers for the right end (sp6 promoter seq. side) detection were pBelo1; 5'-CCGCTCACAATTCCACACA and pBelo2; 5'-CCGGCAGTTTCTACACAT (product size; 600 bp). For typing the T^{137} allele, TmycJ1 and TmycJ2 were used as described previously [1]. For detection of t-haplotype DNA, TCP51, 5'-TGT GAT GAT GAG CTG ATC TTA, and TCP32, 5'-AAA CTC TGC AAT AGC AAG CTG were used. Since the amplified product has a *TaqI* polymorphism, the product from *t*-chromosome

can be cleaved with *TaqI*, while the one from wild type chromosome cannot be digested.

Mice: The mouse strains used for the analysis of the *Brachyury* locus were original *Brachyury* (*T/+*) mutant [6] and t^{w5} (*tct/+*) [4]; both were kind gifts of Dr. H. Fujimoto of Mitsubishi Kagaku Institute of Lifesciences. T^{137}, a transgene-induced mutation of the *Brachyury* locus [1] was also used.

FISH analysis of transgene integration:

FISH analyses were performed according to the method of Watanabe *et al.* [21]. Chromosome spreads were hybridized with fluorescence-labeled BAC DNA probes and counterstained with propidium iodide. FISH signals were detected and photographed with a Nikon Microphoto FXA. Chromosomes were identified according to the R banding standards [20].

Results

BAC DNA purification

In contrast to high copy number plasmids, BAC clones are propagated as one or two copies per cell. To collect sufficient amount of BAC DNA for preparative work, large scale culture is usually needed. Although BAC DNA can be isolated by conventional method for plasmid preparation, there are considerable contaminations of bacterial genomic DNA or protein constituents in the BAC DNA preparation, which hamper subsequent cloning step or transgenic mouse production. Therefore, in general, one or two rounds of CsCl gradient purification of DNA from 1–2 l culture of BAC-containing bacteria have been used for preparative work. Here we tried a simpler procedure for BAC DNA purification adopted from the YAC DNA purification method [17]. Cells containing BAC were embedded in agarose plugs, then treated with detergents and enzymes which remove the cell wall, proteins and other cellular materials, and genomic DNA as well as BAC DNA can be prepared *in situ*. The agarose plugs were loaded onto a pulsed field gel, and BAC DNA can be easily separated from genomic DNA. For linearization of BAC DNA, the *cos* site located within the BAC vector arm can be cleaved with λ-terminase. Linearized BAC DNA is excised from the PFGE gel, and the BAC DNA can be purified after β-agarase digestion followed by Microcon-100 concentration (see Materials and Methods, Fig. 1A). As shown in Fig. 1B, intact BAC DNA of ~190-kb in size was purified by the method described. BAC DNA sufficient for a microinjection experiment (~1.5 μg) was purified from only a 10 ml bacterial culture. BAC DNA purified by this method can be stored at 4°C for at least two weeks before microinjection.

Generation of BAC transgenic mice

Using BAC DNA prepared as described above, we generated 43 founder transgenic mice with 8 different BAC constructs (Table 1). Introduced BAC DNAs were linearized with λ-terminase except for the Vs-Cre construct which was digested with *SalI*. Concentration of BAC DNA for injection is usually adjusted to either 1 or 3 ng/μl, as DNA of 5 ng/μl tended to give lower pups numbers (Table 1). The number of pups obtained after BAC DNA injection were generally lower than that with shorter transgene constructs. However, the integration efficiency or the frequency of transgenic mice among the born animals was similar to that obtained with short transgenic constructs (23.8%; 43 transgenics in 181 pups). The sizes of the injected BAC DNA ranged from 75 kb to 180 kb, but there were no apparent correlations between size of the construct and the integration efficiency, or the integrity of the integrated transgene. When linearized with λ-terminase, the left and right arms of BAC DNA can be detected separately by PCR typing. If both arms are positive by PCR typing, it is likely that the entire BAC insert is integrated into the mouse genome without fragmentation [22]. As shown in Table 1, 29 integrations in 34 informative cases (85.3%) appeared to contain both right and left arms, suggesting that frequency of the fragmentation of the BAC DNA when integrated into the genome was not high with our BAC DNA preparations. Loss of one or both of the arms in subsequent generations was not observed for three transgenic lines tested. FISH analyses were performed for three BAC transgenic lines, and a single integration site of the transgene was observed for each BAC construct (Fig. 2, and not shown). In the case of the TBAC19, the transgene was integrated into chromosome 12. In one line of BAC9, integration was found on chromosome 14, while in the other line the transgene was integrated near the centromeric part of chromosome 3 (Fig. 2).

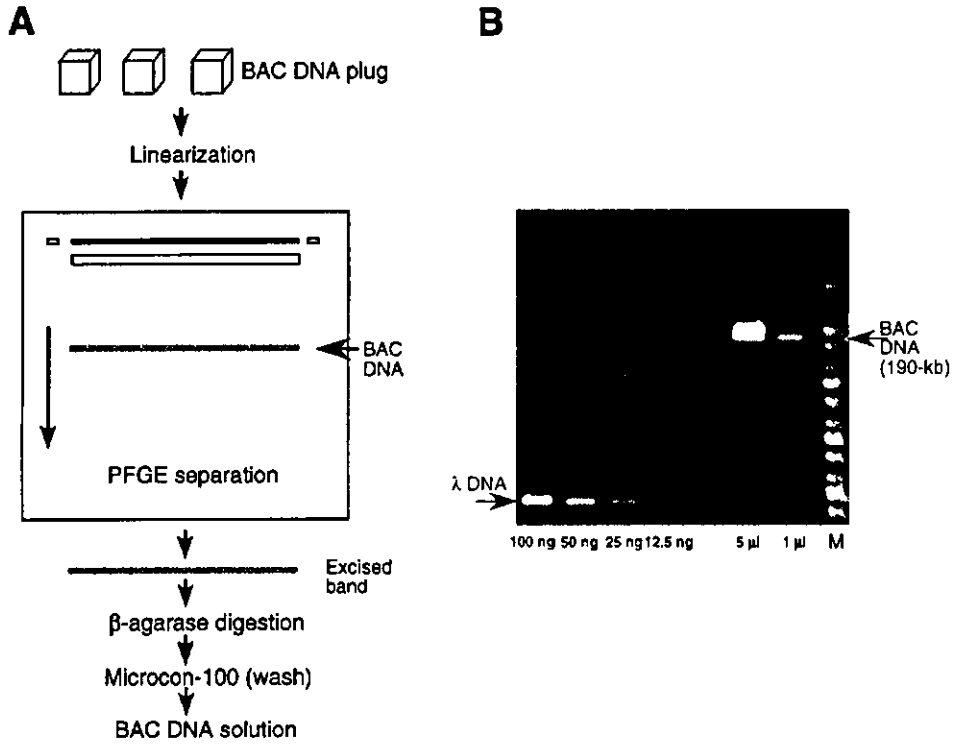

Fig. 1. Purification of BAC DNA for microinjection. A: BAC DNA are prepared in agarose plugs. BAC DNA are linearized by either lamda terminase or rare-cutting restriction enzyme, and separated from *E. coli* genomic DNA by pulsed field gel electrophoresis. A gel slice containing BAC DNA are digested by β-agarase and DNA is purified by a Microcon filtration device. B: BAC DNA purification method described here. A dilution series of lamda DNA was loaded next to the purified BAC DNA. One microliter of BAC DNA corresponded to about 1% of total amount of BAC DNA prepared by the method. M; molecular weight marker.

Functional rescue of Brachyury mutant phenotypes by BAC transgene

We next applied this method for functional characterization of mutations associated with the mouse *T* locus. One line of transgenic mouse harboring a 170-kb fragment of BAC (Fig. 3A) containing the entire *Brachyury* or *T* gene has been established (TBAC19 in Table 1). The copy number of the transgene was estimated to be 1–2 (not shown). The mouse *Brachyury* or *T* gene is required for notochord development and maintenance. Homozygous *T/T* embryos form insufficient axial and paraxial mesoderm and die at 10.5 dpc due to failure in allantois-placental connection [3, 9]. Mice heterozygous for *Brachyury* mutation, *T/+*, exhibit a short tail phenotype, whereas a compound mouse carrying *T* and *tct* (*t*-complex tail interaction), a modifier of tail phenotype, show a tailless phenotype [3]. Stott *et al.* [18] reported that a 23-kb genomic fragment containing the entire *T* gene rescued the short tail phenotype of *T/+*, but failed to rescue the tailless phenotype of T/t^{h51} (*T/tct*). The *T/tct* mouse carrying the 23-kb genomic fragment showed short tail phenotype [10]. Rescue of the embryonic lethal phenotype has not been reported yet. First, we crossed a *T/+* male with a +/+; TBAC transgenic female (Cross 1 in Table 2), and obtained 52 normal tail and 4 short tail offspring. If the BAC transgene is not present, theoretically one half of the offspring would be short tail, *T/+* mice. Therefore, a bias toward normal tail phenotype suggests the possibility that the TBAC transgene complemented the short tail phenotype. If this is the case, out of 52 normal tail mice, 32 were +/+ and 20 were either +/+; TBAC or *T/+*; TBAC. We next randomly selected three normal tail female mice carrying the TBAC transgene, and crossed them with the $t^{w5}/+$ male. In two out of three cases, tailless mice carrying t^{w5} chromosome were obtained

Table 1. List of BAC transgenic lines

BAC clone	size (kb)	conc. (ng/μl)	born pups	integrated total (%)	L arm	R arm
BAC insert prepared by method 1†						
578-N15	130	1,3	27	15 (55.6)	15	14
558-M23	180	5	24	4 (16.7)	4	3
BAC9	165	5	13	2 (15.4)	2	2
TBAC19	170	5	9	1 (11.1)	1	1
BAC132	100	1,3	20	3 (15.0)	3	2
BAC213	75	1,3	14	3 (21.4)	3	3
BAC42	140	1,3	19	6 (31.6)	5	5
*Vs-Cre	140	1,3	55	9 (16.4)	NA	NA
			181	43 (23.8)		
BAC insert prepared by method 2††						
309-M19	140	1,3	19	2 (10.5)	2	1
100-G24	150	1,3	15	1 (6.7)	1	0
			34	3 (8.8)		

*Linearized with *SalI* digestion. Left and right arm of the BAC clones are not present in this case. Others were linearized with λ terminase. NA; not applicable. †Method 1; Insert DNA were prepared in agarose plug, linearized, separated on PFGE and released from agarose by agarase digestion. ††Method 2; BAC DNA was isolated by QIAGEN Large Construct Isolation kit, and circular BAC DNA was injected.

Fig. 2. FISH analysis of BAC transgene integration. A BAC clone isolated from the mouse *quaking* locus (BAC9 in Table 1) was fluorescence-labeled and hybridized to mouse chromosome spreads of a transgenic animal carrying the BAC9 transgene. In this case, the transgene was integrated into chromosome 3 (arrow head).

(Table 2, Cross 1b). The *T* and + allele could not be distinguished in this cross as the classical *T* mutation carries a deletion of > 200 kb at the *T* locus. However, we believe that the genotype of the tailless mice must be T/t^{w5}, since +/+, *T*/+ or t^{w5}/+ mice never show a tailless phenotype [3]. This fact in turn suggests that the genotype of the females 1 and 2 used in Cross 1b were likely to be *T*/+, and that the short tail phenotype of the *T*/+ was complemented by the TBAC transgene. Overrepresentation of normal tail offspring from females 1 and 2 also suggests that tailless phenotype of T/t^{w5}, possibly present in the offspring, were rescued by the TBAC transgene. To obtain further evidence for the functional rescue of the tail phenotype by the BAC transgene, we next used T^{137} allele, a transgene-induced null allele of the *T* locus [1]. The T^{137}/+ heterozygote has a short tail, and shows a tailless phenotype when it is crossed with the complete *t*-haplotype mice such as t^{w5}, which carry the *tct* mutation. Since T^{137} is a transgene-insertional mutation, the genotype can be easily typed with PCR. As shown in Cross 2, the TBAC transgene indeed rescued the short tail phenotype of T^{137}/+. Although +/+; TBAC mice should contain more

Table 2. Genotypes and phenotypes of offspring from crosses between *T/t* mutants and +/+; TBAC

	Tail phenotypes		
	normal	short	absent
Cross 1 (+/+; TBAC × *T*/+)			
	52*	4**	0
Cross 1b (normal tail; TBAC from Cross 1 × t^{w5}/+)			
female 1	9	0	1***
female 2	7	0	1***
female 3	8	0	0
Cross 2 (+/+; TBAC × T^{137}/+)			
Genotype			
+/+	3	0	0
+/+; TBAC	6	0	0
T^{137}/+; TBAC	4	0	0
T^{137}/+	0	10	0
Cross 3 (T^{137}/+; TBAC × *T*/t^{w5})			
Genotype			
T^{137}/t^{w5}; TBAC	6	0	0
T^{137}/T; TBAC	5	0	0
t^{w5}/+; TBAC	5	0	0
t^{w5}/+	1	0	0
T/+; TBAC	1	0	0
T/+	0	2	0
T^{137}/t^{w5}	0	0	3
Cross 4 (T^{137}/t^{w5}; TBAC × T^{137}/t^{w5}; TBAC)			
Genotype			
T^{137}/T^{137}; TBAC	6	0	0
T^{137}/t^{w5}; TBAC	7	0	0
T^{137}/t^{w5}	0	0	4

*Of these 52 normal tail mice, 32 mice did not carry the TBAC transgene, while 20 mice carried the TBAC transgene. Thus these 20 mice can be genotyped as either +/+; TBAC or T/+; TBAC. **These short tail mice can be genotyped as *T*/+, as the *T*/+ mice have a short tail clearly distinguishable from that of the wild-type mice on this genetic background. ***These zero tail mice had the t^{w5} chromosome, thus likely to be *T*/t^{w5}.

than three copies of the *Brachyury* gene, these animals showed normal morphological phenotypes. To test whether the BAC can rescue the tailless phenotype produced by interaction between *T* and *tct*, a *T*/t^{w5} zero tail male was crossed with a T^{137}/+; TBAC female (Cross 3 in Table 2). Three mice genotyped as T^{137}/t^{w5} were tailless as expected. However, six mice with T^{137}/t^{w5} genotype had completely normal tails in the presence of the TBAC transgene, indicating functional correction of the tailless phenotype by BAC. Genotypes of two short tail mice and one normal tail with the BAC transgene are likely to be *T*/+ and *T*/+; TBAC, respectively in this breeding scheme. Therefore it should be noticed that five normal tail mice with the TBAC transgene but not with t^{w5} chromosome could have the T^{137}/T; TBAC genotype, suggesting that the embryonic lethality may also be rescued by the transgene. To investigate this point further, T^{137}/t^{w5}; TBAC male and female mice produced from Cross 3 were mated and their offspring were examined (Cross 4 in Table 2). The results demonstrate that the embryonic lethality of the T^{137}/T^{137} was indeed rescued by TBAC19. Absence of the t^{w5}/t^{w5} or the t^{w5}/t^{w5}; TBAC19 mice can be interpreted by the embryonic lethality of t^{w5} manifested at the gastrulation stage [4]. Recombination between *T* or wild type chromosome and the t^{w5} haplotype was negligible due to chromosome inversion associated with the t^{w5} haplotype [3].

Discussion

More than 40 founder transgenic mice have been produced with BAC DNA prepared by the method described in this paper. There is a tendency for the number of pups to be fewer in comparison with conventional transgenesis when BAC DNA, especially of high concentration, was injected. However, integration frequency in the pups was sometimes even higher than regular transgenesis, and the overall number of transgene integrations was comparable to that obtained with short transgene constructs. Moreover, PCR assay with primers corresponding to right and left arms of linearized BAC DNA revealed that most of (85.3%) the transgenic mice contained intact BAC inserts. In contrast, circular BAC DNA prepared by alkali lysis followed by column purification gave a three-fold lower transgene integration rate and fewer intact transgene integrations. Antoch *et al.* [2] reported that injection of circular BAC DNA yields transgenic offspring at rates comparable to linearized, purified DNA. Thus it should be possible to use circular DNA for microinjection, but there is an argument that undesirable DNA might be generated by random recombination within constructs before integration when circular DNA is used [7].

These data altogether suggest that the method described in this paper could produce linearized BAC DNA with sufficient quantity, purity and integrity for microinjection experiments. Preparation of sufficient amount of intact BAC DNA for microinjection has been

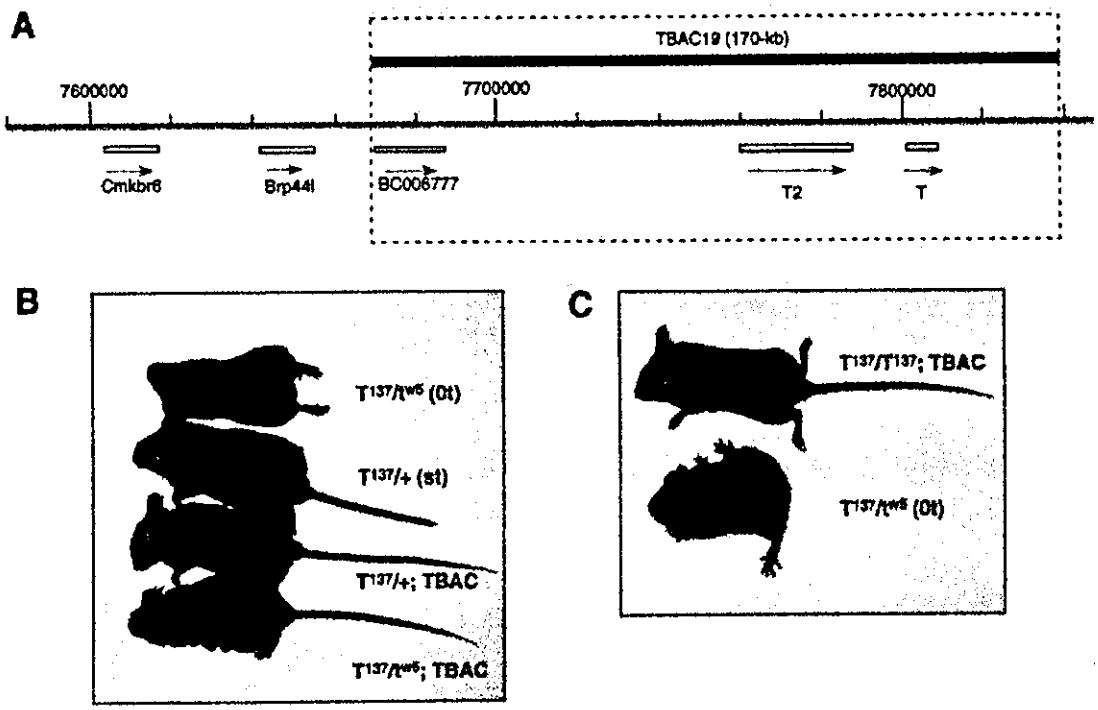

Fig. 3. A: A genomic map around the mouse *T* locus. Numbers shown above the line are nucleotide positions of mouse chromosome 17; this information was retrieved from UCSC mouse genome browser (http://genome.ucsc.edu/). Transcription units mapped in this genomic region are shown under the line: T, *Brachyury*, NM_009309; T2, *Brachyury the Second*, NM_013682; BC006777, Mouse mRNA, similar to CG5104 gene product, BC006777; Brp44l, Brain protein 44 like, NM_018819; Cmkbr6; carbon catabolite repression 6 homolog, NM_009835. Position of TBAC19 used for the rescue experiment was determined by sequencing both ends of the insert and mapping onto the C57BL/6J genome sequence data. B: Tail phenotypes of *T* mutants and rescued animals. T^{137}/t^{w5} showed zero (absent) tail (0t), and $T^{137}/+$ had short tail (st). In contrast, $T^{137}/+$; TBAC and T^{137}/t^{w5}; TBAC mice showed normal tail phenotype. C: Morphology of rescued animal. T^{137}/T^{137}; TBAC had normal tail and normal gross appearance.

a laborious task. BAC DNA isolation protocol usually involves large scale, i.e. one to two liter bacterial culture followed by CsCl density gradient purification and/or column chromatography [5, 24; http://www.med.umich.edu/tamc/BACDNA.html]. The integration efficiency or frequency of transgene fragmentation was not described in detail in these studies. Takahashi *et al.* [22] compared three methods and found that integration of intact DNA occurred only when the BAC DNA was isolated by CsCl gradient centrifugation followed by linearization with restriction enzyme, PFGE separation, and β-agarase digestion. They found that four animals out of twelve transgenic founders (33.3%) possessed both ends of the BAC transgene. In contrast, more than 80% of our transgenic mice carried intact BAC DNA. Therefore our method is apparently simpler and requires less resources and labor, and may serve as a better alternative to the existing protocols.

Stott *et al.* [18] reported that a single copy transgene of a 23-kb genomic fragment containing the entire *T* gene transcription unit could rescue the short tail defect of *T*/+ mice, but failed to complement *T*/*tct* and *T*/*T* phenotypes [10, 18]. The *T*/*tct* mouse carrying the 23-kb genomic fragment showed the short tail phenotype [10]. In contrast, our transgenic mice harboring *T* gene-containing BAC could rescue both short tail phenotypes of *T*/+ and $T^{137}/+$, and the tailless phenotype of T^{137}/t^{w5} (Fig. 3). The TBAC transgene probably rescued the tailless phenotype of the T/t^{w5}, since the majority of the offspring from females 1 and 2 of the Cross 1b in Table 2 exhibited the normal tail phenotype. The *T* allele carries a large (>200 kb) deletion with undefined breakpoints, which covers the entire *T* transcription unit and probably several other genes, while the T^{137} allele