Journal of Reproduction Engineering
Vol.7, Supplement, 308-320, 2005

Analysis of CpG Methylaion of Gene Promoter Regions for Reprogramming of Bovine Reconstructed Embryos

Takashi Kurihara,[1,4*] Kazuya Matsumoto,[2] Aya Kasamatsu,[1] Keiichiro Tominaga,[3] Masayuki Anzai,[2] Tasuku Mitani,[2] Hiromi Kato,[2] Kazuhiro Saeki,[2] Yoshihiko Hosoi,[2] and Akira Iritani[2]

[1] Department of Genetic Engineering, Kinki University Graduate School, Naka-gun, Wakayama 649-6493, Japan
[2] Institute of Advanced Technology, Kinki University, Kainan, Wakayama 642-0017, Japan
[3] Forestry and Fisheries, Hyogo Prefectural Institute for Agriculture, Kasai, Hyogo, 679-0198, Japan
[4] Current address: LA Center, Oriental Yeast Co., Ltd, Tagata-gun, Shizuoka 410-2402, Japan

ABSTRACT

The detail status of CpG methylation in genome of reconstructed embryos is still unclear. In this study, we examined the methylation status of the satellite I region and the promoter regions of the functional genes: telomerase RNA (TR) and interleukin-6 (IL-6) genes in bovine blastocysts produced by nuclear transfer using fetal fibroblasts after serum starvation culture (NT blastocysts). The satellite I region was more methylated in NT blastocysts than that in the in vitro fertilized (IVF) blastocysts (63% vs 46%, $P< 0.05$). In addition, the methylation profiles of the satellite I region in NT blastocysts were similar to that in serum-starved cells, although the profiles were different from that in IVF blasocysts. These results suggest that the highly methylation status of satellite I region in NT blastocysts might indicate the incomplete reprogramming of the genome in embryos reconstructed with somatic cells. While, there were no obvious evidence for aberrant

Received for publication: Dec.1. Accepted: Dec.25
*Correspondence: Dr. T. Kurihara

methylation of the TR and the IL-6 promoter regions of NT blastocysts by comparison with that in IVF blastocysts (0% vs 2.4%: TR and 0% vs 0%: IL-6, P> 0.05). The TR, and the IL-6 genes were expressed in the NT blastocysts as in the IVF blastocysts by RT-PCR analysis.

Key Words: *Bovine reconstructed embryo, CpG methylation, Gene promoter region, Serum starvation, Epigenetic reprogramming*

INTRODUCTION

Successful full-term development of bovine reconstructed embryos following nuclear transfer (NT) with somatic cells into enucleated oocytes has been reported. However, the survival rates of the newborn calves are extremely still low [1-3]. The most of fetuses produced by the NT with somatic cells are absorbed days 30-70, and the most newborn calves have serious developmental abnormalities, resulting in death at birth or shortly after the birth [4-6].

For improvement of developmental capacity of the bovine cloned embryos, many studies have been focused on the cell cycle phase of donor cells and recipient oocytes [7-10]. Culturing donor somatic cells by serum starvation is commonly used for synchronizing the cell cycle before reconstruction of the embryos, because the serum starvation induces a mitotic cell arrest at G0 phase. Although the cell cycle of donor cells have synchronized at G0 phase by the serum starvation culture, little attention has been given to the change of epigenetic modification in the genomes of the donor cells for the NT during the serum starvation culture.

In mammalian genome, the cytosine residue (C) 5' to a guanine residue (G) can form the methylated CpG sequences. This epigenetic modification of DNA in the genome is correlated with gene silencing [11]. Kang et al. [12] examined methylation status of several bovine interspersed nuclear elements including the satellite I region that is the part of sequence in the bovine 1.715 satellite DNA, in embryos reconstructed with somatic cells. The overall genomic methylation status in the reconstructed embryos closely resembled to that in the donor somatic cells, but was very different from that in embryos fertilized in vitro (IVF). Therefore, the low developmental competence of the embryos reconstructed with somatic cells is probably due to incomplete epigenetic reprogramming of the genome [12]. Several reports have

shown that the inappropriate expression of functional genes in reconstructed bovine embryos may result in the low survival rates [13-15]. These inappropriate expressions might be due to incomplete reprogramming in genomes of the reconstructed embryos such as aberrant methylation of gene promoter regions. However, epigenetic reprogramming of the promoter regions of functional genes in donor somatic cells and reconstructed embryos is still unclear.

Telomerase RNA (TR) gene is the RNA component of telomerase that is a ribonucleoprotein reverse transcriptase essential for the maintenance of telomere length controlling the life span of cells [16,17]. In bovine, telomerase activity during early development of reconstructed embryos is needed for restoration of shortened telomere length, which occurred in the genome of the donor cells during the serum starvation culture [18]. In bovine, Interleukin-6 (IL-6) gene begins to be expressed by the blastocyst stage [13]. IL-6 messenger RNA (mRNA) and protein is localized in endometrium at the time of the implantation in human and mice [19,20].

In this study, to reveal the status of reprogramming of promoter regions of functional genes in cultured bovine fibroblasts and bovine blastocysts reconstructed with the cells, we examined the methylation status of the satellite I region as a control, and the promoter regions of TR and IL-6 genes.

MATERIALS AND METHODS

Preparation of cells and NT blastocysts

Skin fibroblasts were isolated from the carcass of a bovine fetus at embryonic day 60 and the cells at passage 5 were used for analyses. At passage 5, the fibroblasts at 60-80% confluency (growing cells) were cultured for further 6 days (serum-starved cells) in Dulbecco's Modified Eagle Medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 0.5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO, USA). Bovine oocytes matured in vitro were used for the recipient cytoplast for NT. In vitro maturation of oocytes was carried out as described previously [21]. After the maturation, both the first polar bodies and the metaphase plates were removed from the in vitro matured oocytes. The enucleated oocytes were individually fused with the serum-starved cells by a single electrical pulse of 2.0 kV/cm for 25 msec. Then, the reconstructed embryos were activated by a single electrical pulse of 200 V/cm for 20 msec and cultured in vitro as described previously [22,23]. For control, IVF embryos were also produced as described previously [24]. Five blastocysts

were collected at 168 h post insemination (IVF blastocysts) or post fusion (NT blastocysts), and then stored at -80 °C until use. The mean number of blastomeres was ~150 and ~100 for IVF and NT blastocysts, respectively.

Isolation of Genomic DNA

For DNA preparation, growing and serum-starved cells (3-4 ´ 10^5 cells) were solubilized in 200 ml of lysis buffer containing 500 mg/ml of proteinase K and 30 mg/ml of RNase A, and then incubate at 37 °C for 16 h. The cellular DNAs were recovered from the lysates by phenol/ chloroform treatment and ethanol precipitation, then dissolved in 150 ml of distilled water (DW) and divided into three groups. While, each 3 group of 5 NT and IVF blastocysts were solubilized in 100 ml of lysis buffer containing 300 mg/ml of proteinase K, then they were incubated at 56 °C for 2 h and at 37 °C for 1 h following addition of RNase A (20 mg/ml). DNAs extracted from the blastocysts were recovered from the lysates by phenol/ chloroform treatment and ethanol precipitation in the presence of 5 mg of glycogen as a carrier, and then dissolved in 50 ml of DW.

Sodium Bisulfite DNA Sequencing

Isolated DNAs were digested with EcoRI at 37 °C for 16 h, then denatured with 0.3 M NaOH at 42 °C for 20 min. Bisulfite modification was initiated by the addition of 520 ml of 3M sodium bisulfite (pH 5) and 30 ml of 10 mM hydroquinone into 55.5 ml of denatured DNA solutions [25,26]. The reaction mixture was covered with mineral oil and incubated at 55 °C for 16 h in the dark. Modified DNA was purified using the Wizard™ DNA purification resin according to the manufacturer's protocol (Promega Co., Madison, WI, USA) and eluted into 50 ml of DW. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 15 min at room temperature, followed by ethanol precipitation. DNAs were resuspended in 20 ml of TE (pH 8.0) and used immediately or stored at -20 °C until use. PCR amplification was carried out using 2 ml of each bisulfite modified DNA as template in 23 ml volume containing PCR buffer (final concentration 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$ and 200 mM of each dNTP), 0.2 mM of each primer and 0.63 U of TaKaRa Taq polymerase Hot Start Version (Takara Bio Inc., Shiga, Japan). We carried out each PCR and pooled the PCR products before sub-cloning. The thermal cycling profiles were performed under the following conditions: 94 °C, 5 min; 40 cycles of 94 °C, 30 sec; 43 °C (for satellite

I region), 46 °C (for TR gene) or 50 °C (for IL-6 gene) 1min; 72 °C, 1min; final extension 72 °C, 7 min. Except for satellite I region, semi-nested PCR was used in other samples to increase the yield of the PCR products. For semi-nested PCR, 2 ml of each first round product were transferred to another 200 ml tube in 23 ml volume containing PCR buffer, each primer and DNA polymerase as above, and amplified for 40 cycles according to the same profiles. The primers used for amplifying the satellite I region were 5'-GGTATTTTTGATTTTA GA-3' for the forward primer and 5'-CCTATAAATA AAATCAACA-3' for the reverse primer. The primers used for amplifying the TR promoter region were 5'-TTTTAAGAGG TGGAAAATA-3' and 5'-TTTAAGTTAA TTTTTAAGTTATAAG-3' for the forward primers, and 5'-TCTCCTTATAAATACAA CAAC-3' for the reverse primer. The primers used for amplifying the IL-6 promoter region were 5'-TTTAAGATATG TTAATGTGTTGAGT-3' and 5'-GTGATT TAGTTTTTGAGGAT TAG-3' for the forward primers, and 5'-AAACAAACTA AATCTCAAACATC-3' for the reverse primer.

Each PCR product was cloned into pGEM-T easy vector (Promega Co.). Individual clones were sequenced using an automatic sequencer ABI PRISM 310.

Isolation of RNA and RT-PCR

Messenger RNA from 5 NT or IVF blastocysts was extracted using QuickPrep micro mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK). Growing and serum-starved cells (3-4 ´ 10^5 cells) after washing with phosphate buffered saline (Gibco BRL, Grand Island, NY, USA) were lysed by the addition of Trizol Reagent (Gibco BRL), and the RNAs were extracted by ethanol precipitation. The total RNAs were resuspended in 20 ml of diethylpyrocarbonate-treated water. Residual DNA in the all extracts was removed by treatment with 0.3 U / ml DNase I (Takara Bio Inc.). RT-PCR was performed using a Superscript One-step PCR Kit (Gibco BRL) according to the manufacture's protocol. Briefly, the RNA samples were pre-incubated at 65 °C for 5 min. RT-PCR was then performed at 50 °C for 30 min, 94 °C for 2 min, 40 cycles of amplification (94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec), and 72 °C for 7 min after addition of 0.5 ml RT/platinum Taq mixture, 12.5 ml of 2 ´ reaction mixture, 10 U ribonuclease inhibitor (Takara Bio Inc.) and 0.2 mM each sense and reverse primers to 1 ml of the RNA samples. Reactions were performed with forward 5'-CGCTGTTTTTCTCGCTGACTTTC-3' and reverse5'-CTGACAGAGCCCAATTC TTCACG-3' primers to detect TR cDNA,

and forward 5'-CAGGAACAGCTATGAA CTCC-3' and reverse 5'-TTGCAGAGA TTTTGTCGACC-3' primers to detect IL-6 cDNA. Control detection of glyceraldehyde -3-phosphate dehydrogenase (GAPDH) was performed with forward 5'-ACCAC AGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3' primers [27].

Statistical Analysis

The data on the methylation rates in cells and blastocysts were analyzed by ANOVA and Fisher's protected least significant difference test using the STATVIEW program ver. 5 (Abacus Concepts, Berkeley, CA). Data were expressed as mean±SEM. The level of significance was set at $P<0.05$.

Note

The sequences of mRNA and genomic DNA encoding bovine TR, IL-6, G3PDH, satellite I region and promoter regions of TR and IL-6 genes have been assigned nucleotide accession number: AF054814, X57317, U85042, V00125, AF121226 and Z11749, and respectively.

RESULTS

We first analyzed the methylation status of satellite I region [12] in growing and serum-starved fibroblasts and NT and IVF blastocysts. Methylation rates were not significantly different between the growing and the serum-starved cells and methylation status was also relatively homogenous among different clones (50-100%, Fig. 1A). The methylation rate of satellite I region in NT blastocysts was significantly lower than that in serum-starved cells (63% vs 80%, $P< 0.05$, Fig. 1A), even though NT blastocysts were produced from the same population of the serum-starved cells. We also found that the satellite I region in the NT blastocysts was significantly methylated in contrast to that in the IVF blastocysts (63% vs 46%, $P<0.05$, Fig. 1A). The sequences of CpG-3 and CpG-6 showed the lowest and the highest methylation levels, respectively, in both the NT blastocysts and the serum-starved cells (Fig. 1B).

We next analyzed the metylation status of 16 CpG dinucleotides immediately upstream of the TR gene containing the promoter region up to -231 bp from -19 bp far from transcription start site (designated as+1) in cells and embryos. Figure 2A showed that, except for the IVF blastocysts, 16 CpG dinucleotides in the other 3 groups were all demethylated. The -61 CpG sequence of 3 clones and the -44 CpG sequence of 8 clones were specifically methylated in IVF blastocysts (Fig. 2A). By comparing with 16 CpG dinucleotides

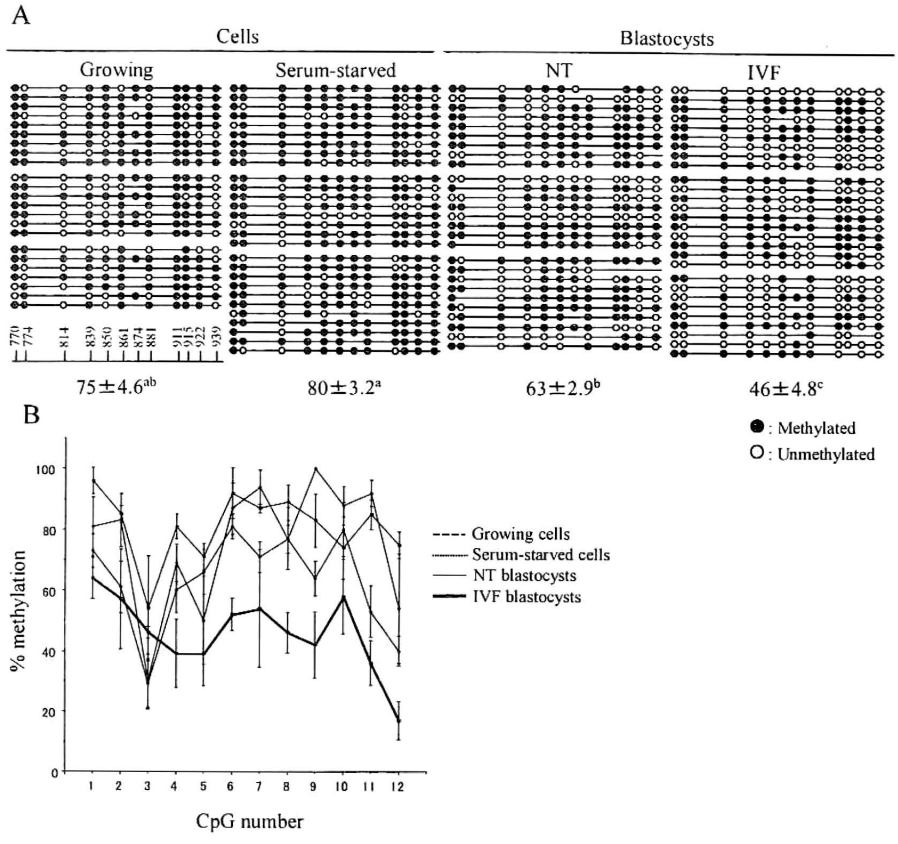

Fig. 1. DNA methylation status of *satellite I* region in genomic DNAs using sodium bisulfite DNA sequencing method. A) DNA methylation profiles of the individual CpG site at the *satellite I* region in growing cells, serum-starved cells, NT blastocysts and IVF blastocysts. Bottom figure of the growing cells indicates the CpG dinucleotide location in the 1399 bp repeated unit of bovine *1.715 satellite DNA*. Methylation rates of each sample have shown to bottom of each methylation profile. Unmethylated status (open circle) and methylated status (close circle) is indicated at each CpG sequence. Each PCR product was sub-cloned, and 23 to 28 clones (7 to 11 clones × 3) subjected to nucleotide sequence analysis. In the *satellite I* region, 12 CpG dinucleotides were hypermethylated in the growing cells and the serum-starved cells. The *satellite I* region of the NT blastocysts was significantly hypermethylated than that of the IVF blastocysts. a-c: Different letters denotes significant difference among the methylation rates ($P<0.05$). B) Methylation profile of growing cells (broken line), serum-starved cells (dotted line), NT blastocysts (thin line) and IVF blastocysts (thick line) at 12 CpG dinucleotides. The sequences of CpG-3 and CpG-6 showed the lowest and the highest methylation levels, respectively, in both the NT blastocysts and the serum-starved cells.

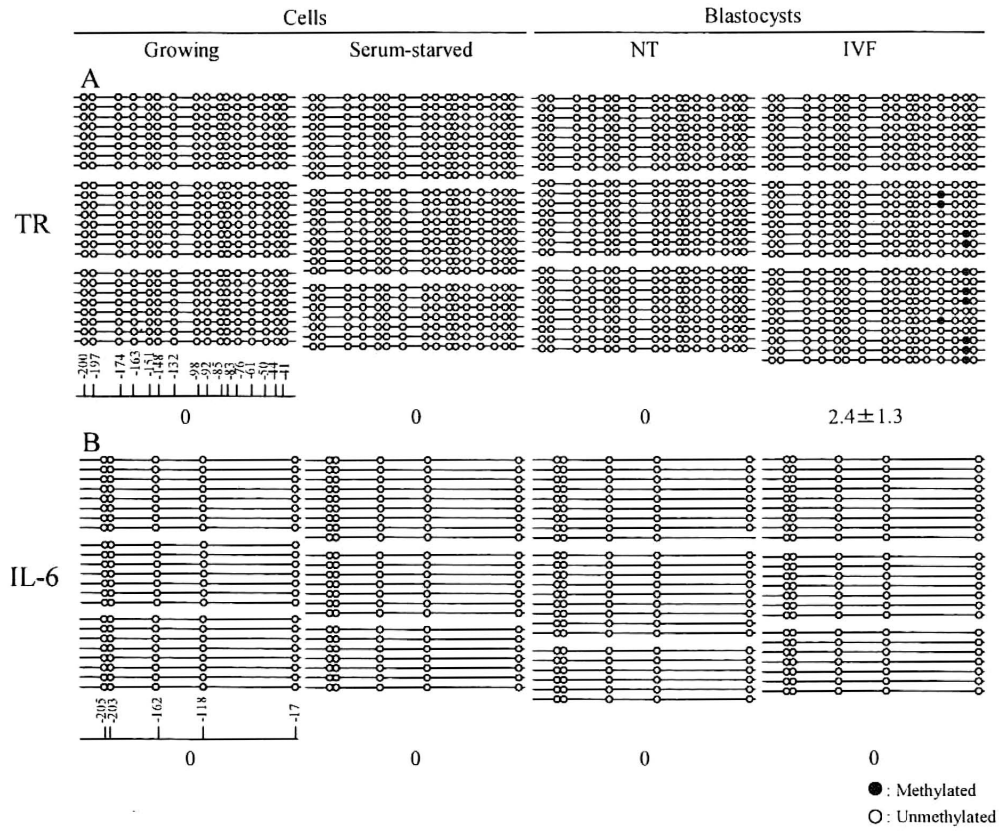

Fig. 2. DNA methylation status of *TR* gene and *IL-6* gene promoter regions. Methylation rates of each sample have shown to bottom of each methylation profile. Bottom figure of the growing cells indicates the CpG dinucleotide location from the transcription start site. Unmethylated status (open circle) and methylated status (close circle) is indicated at each CpG site. A) Methylation profiles of the individual CpG site at the *TR* gene upstream region in growing cells, serum-starved cells, NT blastocysts and IVF blastocysts. Each PCR product was sub-cloned, and 23 to 26 clones (7 to 10 clones × 3) subjected to nucleotide sequence analysis. Sixteen CpG dinucleotides were all demethylated in the growing cells, the serum-starved cells and the NT blastocysts. The -61 CpG sequence of 3 clones and the -44 CpG sequence of 8 clones were specifically methylated in IVF blastocysts. B) Methylation profiles of the individual CpG site at the *IL-6* gene upstream region as the *TR* gene promoter region. Each PCR product was sub-cloned, and 22 to 24 clones (6 to 9 clones × 3) subjected to nucleotide sequence analysis. Five CpG dinucleotides were all demethylated in all 4 groups.

of the other 3 groups, statistical difference of methylation rate in the NT blastocysts was not observed (Fig. 2A). We further analyzed the methylaton status of 5 CpG dinucleotides immediately upstream of the IL-6 gene containing the promoter region up to -253 bp from +9 bp far from transcription start site (designated as +1) in cells and embryos. The 5 CpG dinucleotides in the 4 groups were all demethylated (Fig. 2B). By comparing with 5 CpG dinucleotides of the other 3 groups, statistical difference of methylation rates in the NT blastocysts was not observed (Fig. 2B).

In addition, we examined the expression of TR and IL-6 genes in cells and embryos by the RT-PCR method. Transcripts of the TR and the IL-6 genes in all groups were detected (Fig. 3).

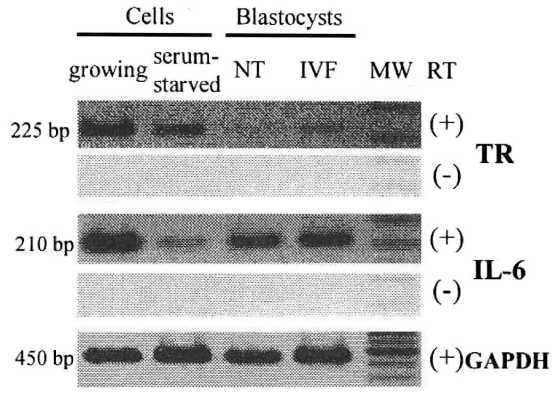

Fig. 3. The expression of TR and IL-6 genes mRNAs in bovine fibroblasts and blastocysts. The expression of the TR, the IL-6, and the GAPDH genes mRNAs were determined by RT-PCR from RNAs of growing cells, serum-starved cells, NT blastocysts and IVF blastocysts. Both positive (+) samples and negative control reactions (-) omitting the RT enzyme are shown in the TR and the IL-6 genes. The DNA ladder 100-bp marker (indicated MW) was used to show the size of PCR products. All 4 RNAs were positive for the TR, the IL-6, and the GAPDH genes expression

DISCUSSION

We analyzed the methylation status of satellite I region in cells and blastocysts as an index of genome wide methylation status. Our result for the satellite I region was almost as same as a result of Kang et al. [12]. We found that the mathylation rates of the satellite I region were not significantly different between the NT blastocysts and the serum-starved cells., and methylation status suggesting that there were no substantial variations in methylation status of the region of the cells by serum starvation. In addition, methylation rate of the satellite I region in the NT blastocysts was significantly lower than that in the serum-starved cells (63% vs 80%, $P< 0.05$, Fig. 1A) as a report of Kang *et al.* [12]. These results suggest that genome-wide methylation status may be changed by the reconstruction of embryos. These changes might result in reprogramming in the genome of reconstructed embryos. However, the region in the NT blastocysts was significantly more methylated than that in the IVF

blastocysts (63% vs 46%, $P<0.05$, Fig. 1A). The methylation profile of the region in NT blastocysts was similar to that in serum-starved cells, although it was very different from that in IVF blastocysts (Figs. 1B). These results indicate that imcomplate epigenetic reprogramming has occurred in the genome of reconstructed embryos.

TR and IL-6 promoter regions might play important roles in its expression in either pre- or during implantation during bovine development. We found almost no methylated CpG dinucleotides of the TR and the IL-6 promoter regions in growing and serum-starved cells, and in NT and IVF blastocysts in cattle (Figs. 2A and 2B), indicating that there was no obvious evidence for aberrant methylation in the two promoter regions which are need from early developmental stage in reconstructed embryos. The hypomethylation status of the promoter regions of the genes (Figs. 2A and 2B) might result in active expression of the genes, since transcripts of these two genes were detected in the NT blastocysts as in the IVF blastocysts by the RT-PCR analysis (Fig. 3).

In conclusion, our findings indicate that there was no obvious evidence for aberrant methylation in the TR and the IL-6 promoter regions of the reconstructed embryos. However, the incomplete reprogramming of the satellite I region in NT embryos was indicated by comparison with that in donor cells and in IVF embryos. The detail analysis for methylation of genomic DNA in donor cells and reconstructed embryos has major significance in the study of epigenetic reprogramming of donor genome.

ACKNOWLEDGMENTS

This study was supported by a grant-in-aid of the 21st Century Center of Exellence Program of Education, Science, Sports and Culture and for Scientific Research (No.15380196) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and by a grant from the Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence of the Japan Science and Technology Corporation.

REFERENCES

1) Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* 280, 1256-1258, 1998.
2) Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. *Science* 282, 2095-2098, 1998.
3) Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *J. Reprod. Fertil.* 120, 231-237, 2000..
4) Edwards JL, Schrick FN, McCracken MD, van Amstel SR, Hopkins FM, Welborn MG, Davies CJ. Cloning adult farm animals: a review of the possibilities and problems associated with somatic cell nuclear transfer. *Am. J. Reprod. Immunol.* 50, 113-123, 2003.
5) Shi W, Zakhartchenko V, Wolf E. Epigenetic reprogramming in mammalian nuclear transfer. *Differentiation* 71, 91-113, 2003.
6) Han YM, Kang YK, Koo DB, Lee KK. Nuclear reprogramming of cloned embryos produced in vitro. *Theriogenology* 59, 33-44, 2003.
7) Korfiatis N, Trounson A, Lacham-Kaplan O. Cell synchronization for the purposes of nuclear transfer in the bovine. *Cloning Stem Cells* 3, 125-138, 2001.
8) Cho JK, Lee BC, Park JI, Lim JM, Shin SJ, Kim KY, Lee BD, Hwang WS. Development of bovine oocytes reconstructed with different donor somatic cells with or without serum starvation. *Theriogenology* 57, 1819-1828, 2002.
9) Katska L, Bochenek M, Kania G, Rynska B, Smorag Z. Flow cytometric cell cycle analysis of somatic cells primary cultures established for bovine cloning. *Theriogenology* 58, 1733-1744, 2002.
10) Wells DN, Laible G, Tucker FC, Miller AL, Oliver JE, Xiang T, Forsyth JT, Berg MC, Cockrem K, L'Huillier PJ, Tervit HR, Oback B. Coordination between donor cell type and cell cycle stage improves nuclear cloning efficiency in cattle. *Theriogenology* 59, 45-59, 2003.
11) Ogawa H, Ono Y, Shimozawa N, Sotomaru Y, Katsuzawa Y, Hiura H, Ito M, Kono T. Disruption of imprinting in cloned mouse fetuses from embryonic stem cells. *Reproduction* 126, 549-557, 2003.

12) Kang YK, Koo DB, Park JS, Choi YH, Chung AS, Lee KK, Han YM. Aberrant methylation of donor genome in cloned bovine embryos. *Nat. Genet.* 28, 173-177, 2001.

13) Daniels R, Hall V, Trounson AO. Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. Biol. Reprod. 63, 1034-1040, 2000.

14) Daniels R, Hall VJ, French AJ, Korfiatis NA, Trounson AO. Comparison of gene transcription in cloned bovine embryos produced by different nuclear transfer techniques. *Mol. Reprod. Dev.* 60, 281-288, 2001.

15) Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R, Niemann H. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. Biol. Reprod. 65, 309-317, 2001.

16) Blackburn E, Gilley D, Ware T, Bhattacharyya A, Kirk K, Wang H. Studying the telomerase RNA in Tetrahymena. *Methods Cell Biol.* 62, 417-432, 2000.

17) Keith WN, Sarvesvaran J, Downey M. Analysis of telomerase RNA gene expression by in situ hybridization. *Methods Mol. Biol.* 191, 65-81, 2002.

18) Betts D, Bordignon V, Hill J, Winger Q, Westhusin M, Smith L, King W. Reprogramming of telomerase activity and rebuilding of telomere length in cloned cattle. *Proc. Natl. Acad. Sci. U S A* 98, 1077-1082, 2001.

19) Meisser A, Cameo P, Islami D, Campana A, Bischof P. Effects of interleukin-6 (IL-6) on cytotrophoblastic cells. *Mol. Hum. Reprod.* 5, 1055-1058, 1999.

20) Desai N, Scarrow M, Lawson J, Kinzer D, Goldfarb J. Evaluation of the effect of interleukin-6 and human extracellullar matrix on embryonic development. *Hum. Reprod.* 14, 1588-1592, 1999.

21) Saeki K, Nagao Y, Kishi M, Nagai M. Developmental capacity of bovine oocytes following inhibition of meiotic resumption by cycloheximide or 6-dimethylaminopurine. *Theriogenology* 48, 1161-1172, 1997.

22) Akagi S, Adachi N, Matsukawa K, Kubo M, Takahashi S. Developmental potential of bovine nuclear transfer embryos and postnatal survival rate of cloned calves produced by two different timings of fusion and activation. *Mol. Reprod. Dev.* 66, 264-272, 2003.

23) Akagi S, Takahashi S, Adachi N, Hasegawa K, Sugawara T, Tozuka Y, Yamamoto E, Shimizu M, Izaike Y. In vitro and in vivo developmental potential of nuclear transfer

embryos using bovine cumulus cells prepared in four different conditions. *Cloning Stem Cells* 5, 101-108, 2003.

24) Saeki K, Hoshi M, Leibfried-Rutledge ML, First NL. In vitro fertilization and development of bovine oocytes matured in serum-free medium. *Biol. Reprod.* 44, 256-260, 1991.

25) Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* 22, 2990-2997, 1994.

26) Grunau C, Clark SJ, Rosenthal A. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucleic Acids Res.* 29, E65-5, 2001.

27) Kaneko K, Saeki K, Matsumoto K, Nakagami K, Hosoi Y, Kato H, Iritani A. Detection of messenger ribonucleic acid coding for glyceraldehydes- 3-phosphate dehydrogenase in single bovine oocytes and early embryos. *J. Manm. Ova Res.* 16, 11, 1999.