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DETERMINATION OF THE ABSOLUTE NUMBER OF TRANSGENE COPIES IN CMVFUT TRANSGENIC PIGS*

Daniel Lipiński[1,2], Joanna Zeyland[1], Andrzej Pławski[2], Ryszard Słomski[1,2]

[1]Department of Biochemistry and Biotechnology, University of Life Sciences, Wołyńska 35, 60-637 Poznań, Poland
[2]Institute of Human Genetics, Polish Academy of Sciences, Strzeszyńska 32, 60-479 Poznań, Poland

Abstract

The aim of this research was to determine the number of transgene copies in the DNA of transgenic pigs. The copy number of the transgene was analysed in the transgenic animals with introduced pCMVFUT genetic construct containing a coding sequence of human H transferase under a control of CMV promoter. The copy number of the transgene that had integrated with the genome of the transgenic animals was analysed by qPCR with SYBR Green dye, which enabled nonspecific double-stranded DNA detection. CMVFT-2F and CMVFT-2R primers were used to amplify a 149 bp fragment of DNA. Forward primer had a sequence complementary to a promoter sequence and reverse primer to a coding sequence of H transferase. The copy number of the transgene in the examined samples was established by plotting the C_T values obtained on a standard curve, which had been set by the usage of the C_T values for the successive standard dilutions with known copy number (1.43^8–1.43^1 copies). As a standard we used pCMVFut genetic construct hydrolyzed with *Not*I restriction enzyme to a linear form. The real-time PCR results helped to establish the range of 3 - 4 as the number of the transgene copies that had integrated to the swine genome.

Key words: animal transgenesis, RT-PCR, transgene copy number

Exogenic DNA may integrate with genomic DNA by a homologous recombination whose mechanism is based on the sequence similarity between the integrating DNA and genomic DNA or via non-homologous integration. The non-homologous integration of exogenic DNA makes it possible to modify genetic information of a cell leading to the development of transgenic animals. Prior to their integration into the genome, fragments of exogenic DNA participate in the process of homologous recombination occurring between transgene fragments (Folger et al., 1985) leading to the formation of the so called concatamers (Folger et al., 1982), i.e. serially con-

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nected transgene particles. The incorporation of such a structure into the genome may exert a negative impact on the content, organization and functioning of the genome. The numbers of transgene copies which are built into the genome as well as the place of integration also affect the level of its expression.

Traditionally, the number of transgene copies in transgenic animals was analysed using hybridization methods (Southern blot, point hybridization) but these methods were labour- and time-consuming and they required relatively large quantities of DNA for analyses. At present, hybridization methods are being replaced by a faster, cheaper and simpler real-time PCR method. Real-time PCR found numerous applications in the analysis of genetically modified organisms, among others, to determine the amount of copies incorporated into the genome (Ballester et al., 2004; Ingham et al., 2001) or zygosity of the examined animals (Tesson et al., 2002; Shitara et al., 2004).

The aim of this research was to determine the number of transgene copies in the DNA of transgenic pigs with introduced additional copies of a human gene encoding H transferase (Jura et al., 2004). The expression of this gene leads to a reduction of Gal epitope number on a surface of animal cells (Lipiński et al., 2010). Gal epitope is responsible for an organ rejection in swine-primates transplantation set. The transplantation of animal derived organs could save thousands of human beings who do not undergo transplant surgery of heart, kidney or liver because of the lack of donors. All intensive and increasing studies which could help to solve this problem are focused on pigs. These animals are easy and cheap to breed, multiply fast and have a lot of progeny. The diversification between breeds allows the organs' selection of an appropriate size for the recipients. Pigs and humans have similar anatomical and physiological parameters, but the significant genetic distance is among the main causes of the major immunological complications after organ grafting. Organs are rejected almost immediately after transplantation as result of a hyperacute rejection (HAR). The main reason of HAR is the presence of xenoreactive antibodies in humans against pig Gal antigens present on glycolipids and glycoproteins. Gal antigen (Galα1,3Gal) develops from α1,3-galactosyltransferase which adds galactose to N-acetyllactosamine (N-lac) with α1,3-glycosyl bond (Sandrin and McKenzie, 1994). Both the enzyme and the sugar residue are not present in humans and Old World monkeys (Galili et al., 1988). It is assumed that the gene encoding α1,3-galactosyltransferase was inactivated in ancestors of higher primates (Galili and Swanson, 1991). Introduction of additional copies of gene encoding human H transferase into a pig genome could prevent an immunological response. This enzyme catalyzes fucose addition to N-lac forming a neutral H structure. The mechanism involved in the reduction of Gal epitope as a result of H transferase expression is based on the competition between H transferase and α1,3-galactosyltransferase for the same acceptor substrate. The introduction of additional copies of H transferase would result in reduced number of Gal antigens on the surface of donor cells and lead to a decrease in immunogenicity (Sharma et al., 1996; Costa et al., 1999). In this paper we present the evaluation of the number of transgene copies in DNA of transgenic pigs with introduced additional copies of a human gene encoding H transferase by real-time PCR. A knowledge about the number of transgene copies integrated with host

DNA is an important premise in inferring the level of the transgene expression and in a selection of the transgenically valuable individuals.

Material and methods

The number of transgene copies was analysed in transgenic pigs obtained by microinjection of the pCMVFut construct into the pronuclei of male fertilized egg cells (Jura et al., 2004). The pCMVFut gene construct contained a systemic CMV-IE regulatory sequence (618 bp), cDNA of the gene encoding human H (HT) transferase (1098 bp) as well as a signal sequence of the poly(A) human GH gene (104 bp) introduced into the plasmid pGT-N29 vector (New England BioLabs) (4662 bp). Analyses were performed on selected homozygous transgenic animals (433, 437) as well as on heterozygous (431, 435) and non-transgenic animals. DNA was isolated from the animal ear bioptates employing the method with K proteinase. 680 µl SE buffer (75 mM NaCl, 1 mM EDTA, pH 8.0), 20 µl K proteinase (10 mg/ml) and 100 µl 10% SDS were added to 0.1–0.2 g of tissue. Samples were mixed thoroughly and incubated at 55°C for 16 hours. The lysate obtained was then supplemented with phenol:chloroform:isoamyl alcohol (50:49:1) and mixed intensively. Samples were centrifuged at 2000x g for 5 minutes and the liquid phase was collected. Next, 1 ml of chloroform was added and samples were again mixed and centrifuged and then one volume of isopropanol was added to one liquid phase, the pellet was collected, and rinsed with 1 ml 70% ethanol. Next, the pellet was collected again, dried and dissolved in 100 µl water.

Oligonucleotide primers synthesized by OLIGO Company were used in the reaction. They were designed with the assistance of the Primer3 program. Employing the following pair of CMVFT-2F (5'- TGG GAG GTC TAT ATA AGC AGA G -3') and CMVFT-2R (5'- GAA GAT TAC AGA GAG GAC ACA G -3'), a 149 bp long fragment of a transgene was amplified. The F primer was complementary to the CMV promoter sequence, while the R primer was complementary to the sequence encoding H transferase.

The real-time PCR reaction was performed employing equipment designed for real-time PCR *Brilliant® II QPCR Master Mix* (Stratagene) and the Mx3005P apparatus (Stratagene). 25 µl of the reaction mixture contained 4 pM CMVFT-2F and CMVFT-2R primers, 1x concentrated *Master Mix* and 30 ng of genomic DNA. The absolute numbers of the transgene copies in the examined sequences were determined by comparing the amplification results with the standard curve established on the basis of CT values obtained for consecutive standard dilutions of known numbers of copies (1.43^{8}–1.43^{1} copies) (pCMVFut gene construction hydrolyzed with the restriction enzyme *Not*I). The qPCR reaction was conducted under the following conditions: initial denaturation 95°C, 600 sec.; denaturation 95°C, 20 sec.; annealing 58°C, 30 sec.; elongation 72°C, 30 sec.; 32 cycles. The results were analysed with the assistance of the *MxPro QPCR Software for Mx3000P and Mx3005P QPCR Systems version 3.20* (Stratagene) software. The number of transgene copies in the

examined samples was established by plotting the CT values obtained onto the standard curve. In order to minimize the error associated with the different quantity of the applied template, the analysis was repeated three times and the results obtained were averaged.

Results

Since 1 ng of DNA corresponds to the molecular mass of 606060600 MDa and the molecular mass of the swine genome amounts to 1820000 MDa, therefore 1 ng of swine genomic DNA contains approximately 333 copies of the genome. 15 ng of swine genomic DNA were used for each qPCR reaction, hence each of the examined samples contained about 4995 copies of the swine genomic DNA. In order to calculate the number of copies of the transgene incorporated into a single genome of transgenic animals, the absolute number of copies of the transgene in the examined sample was divided by the number of copies of the genomic DNA. In the case of heterozygotic transgenic animals, the transgene occurs only on one of the pair of homologue chromosomes, therefore in order to calculate the number of transgene copies built into one of the chromosomes the calculation results obtained were multiplied by 2. The results of the real-time PCR analysis allowed estimating the number of transgene copies incorporated into the swine genome at the interval of 3.2 to 4.05 (Table 1). As expected, no amplification of the PCR product was observed in the case of non-transgenic animals as well as in the negative control (without DNA) (Fig. 1).

Table 1. Results of real-time PCR analysis using Mx3005P (Stratagene) apparatus. Samples 1–8 – standard (1.43^8–1.43^1 copies of the pCMVFut gene construction); samples 9–10 – non-transgenic animals (5C, 1B); 11–12 – homozygotic transgenic animals (433, 437); samples 13–15 – heterozygotic transgenic animals (431, 435, TG1154); sample 16 – negative control (without DNA)

Number	Type	Ct (dR)	Number of transgene copies in analysed sample	Number of transgene copies in genome	Tm products (-R'(T))
1	Standard	8.57	1.43e+008	-	84.56
2	Standard	12.00	1.43e+007	-	84.57
3	Standard	16.19	1.43e+006	-	84.57
4	Standard	19.94	1.43e+005	-	84.57
5	Standard	21.55	1.43e+004	-	84.57
6	Standard	25.19	1.43e+003	-	84.57
7	Standard	28.07	1.43e+002	-	84.57
8	Standard	31.99	1.43e+001	-	84.57
9	5C	No Ct	No Ct	-	-
10	1B	No Ct	No Ct	-	-
11	433	21.53	2.026e+004	4.05	84.56
12	437	21.63	1.893e+004	3.79	84.56
13	431	22.85	7.980e+003	3.2	84.56
14	435	22.54	9.959e+003	3.99	84.56
15	TG1154	22.63	9.316e+003	3.79	84.10
16	NTC	No Ct	No Ct	-	-

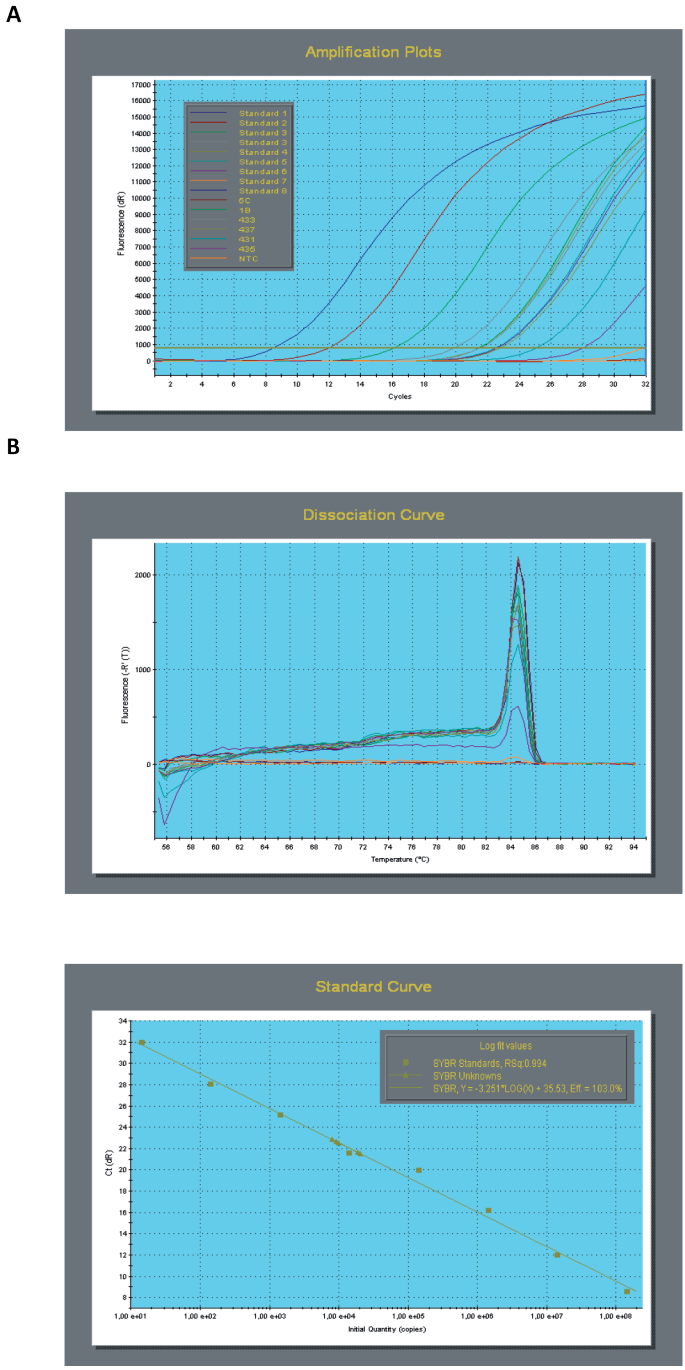

Fig. 1. Results of real-time PCR analysis using Mx3005P (Stratagene) apparatus.
A – amplification curves; B – dissociation curves; C – standard curve

Discussion

In spite of twice higher number of transgene copies in the homozygotic animals, our previous results of the flow cytometry analysis revealed no significant differences between Gal epitope level achieved by transgene in neither hetero- nor homozygous pigs (Lipiński et al., 2010). The flow cytometry results were confirmed by the cytotoxicity assay. We found no statistical difference in the survival rate between transgenic homozygous or heterozygous cells under the influence of 50% human serum with active complement system. Both homozygous and heterozygous cells had the same level of lysis protection (Lipiński et al., 2010). Despite a seemingly stable incorporation of the transgene into the chromosome, silencing of the transgene expression is a frequent phenomenon taking place in connection with the integration of the exogenous DNA in mammalian cells. It was discovered that the site of incorporation undergoes *de novo* methylation (Muller et al., 2001) which can probably be interpreted as a specific defence of the cell against exogenous DNA. Repeat sequences are especially sensitive to changes during methylation (Muller et al., 2001). As exogenous DNA integrates most frequently in the form of tandem series, it leads to their methylation and blocking of a transgene expression. The level of a transgene expression declines when the number of transgene copies in the tandem series increases (Milot et al., 1996). Investigations carried out so far have failed to explain satisfactorily whether methylation is stimulated by the number of transgene copies or whether it depends on the position of a transgene in a chromosome. In addition, it was also proved that methylation of such transgenic series may lead to localized chromatin formation (Keshet et al., 1986). The following three phenomena coincide at this site: repeatability of the sequences (the transgene tandem series), methylation, and heterochromatinization. Determination of mutual cause and effect relationships between them would be extremely important to elucidate the mechanisms controlling the integration course of the exogenous sequences with genomic DNA and to predict results of their integrations. The employment of the real-time PCR technique to gather information about the number of transgene copies integrating with the genome of modified animals will contribute to faster understanding of these mechanisms. In turn, a better understanding of the mechanisms of non-homologue integration would also make it possible to reduce the negative impact of this phenomenon on the cell genome and would be particularly important for gene therapy.

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DANIEL LIPIŃSKI, JOANNA ZEYLAND, ANDRZEJ PŁAWSKI, RYSZARD SŁOMSKI

Określenie liczby kopii transgenu u transgenicznych świń CMVFut

STRESZCZENIE

Celem pracy było określenie liczby kopii transgenu w DNA transgenicznych świń. Liczbę kopii transgenu analizowano u zwierząt transgenicznych z wprowadzoną konstrukcją genową zawierającą sekwencję kodującą transferazy H pod kontrolą promotora CMV. Liczbę kopii transgenu, jaka wbudowała się do genomu zwierząt transgenicznych analizowano metodą qPCR z zastosowaniem barwnika SYBR Green umożliwiającego nieswoistą detekcję dwuniciowego DNA. Za pomocą pary starterów

CMVFT-2F i CMVFT-2R amplifikowano fragment o wielkości 149 pz. Starter F był komplementarny do sekwencji promotora CMV, a starter R był komplementarny do sekwencji kodującej transferazę H. Liczbę kopii transgenu w badanych próbkach ustalano przez naniesienie uzyskanych wartości CT na krzywą standardową, która została wykreślona na podstawie wartości CT, uzyskanych dla kolejnych rozcieńczeń standardu o znanej liczbie kopii ($1{,}43^{8}$–$1{,}43^{1}$). Jako standard zastosowano konstrukcję genową pCMVFut hydrolizowaną do formy liniowej enzymem restrykcyjnym *Not*I. Wynik analizy PCR w czasie rzeczywistym pozwolił określić liczbę kopii transgenu wbudowanego do genomu świni pomiędzy 3 a 4.