Original Article

Localization of Human Coagulation Factor VIII (hFVIII) in Transgenic Rabbit by FISH-TSA: Identification of Transgene Copy Number and Transmission to the Next Generation

(rabbit / hFVIII / transgenesis / FISH-TSA)

V. KRYLOV[1], T. TLAPÁKOVÁ[1], J. MÁCHA[1], J. CURLEJ[2,3], L. RYBAN[4], P. CHRENEK[2,5]

[1]Charles University in Prague, Faculty of Science, Department of Cell Biology, Prague, Czech Republic
[2]Slovak Agricultural Research Centre, Research Institute of Animal Production, Department of Genetics and Animal Reproduction, Nitra, Slovak Republic
[3]Faculty of Biotechnology and Food Science, Slovak Agricultural University, Nitra, Slovak Republic
[4]Department of Vascular Biology and Thrombosis Research, Medical University of Vienna, Vienna, Austria
[5]Department of Zoology and Anthropology, Constantine the Philosopher University, Nitra, Slovak Republic

Abstract. For chromosomal localization of the *hFVIII* human transgene in F2 and F3 generation of transgenic rabbits, FISH-TSA was applied. A short cDNA probe (1250 bp) targeted chromosomes 3, 7, 8, 9 and 18 of an F2 male (animal 1-3-8). Two transgenic offspring (F3) revealed signal positions in chromosome 3 and chromosomes 3 and 7, respectively. Sequencing and structure analysis of the rabbit orthologous gene revealed high similarity to its human counterpart. Part of the sequenced cDNA (1310 bp) served as a probe for FISH-TSA analysis. The rabbit gene was localized in the q arm terminus of the X chromosome. This result is in agreement with reciprocal chromosome painting between the rabbit and the human. The presented FISH-TSA method provides strong signals without any interspecies reactivity.

Introduction

Transgenic rabbits are widely used as bioreactors in producing recombinant proteins and in the study of human diseases (Bozse et al., 2003). In addition, rabbit has become a favorite model organism for the study of gene expression and regulation of human recombinant proteins (Fan and Watanabe, 2003). One of these is human coagulation factor VIII. This protein is deficient in X-linked bleeding haemophilia A, occurring in one of 10,000 males. The condition is treated by supplying human plasma pooled hFVIII concentrates or applying rhFVIII protein obtained through recombinant techniques. Besides cell culture systems producing transgenic protein into the cultivation medium, a method employing transgenic animals producing hFVIII via mammary gland into pig, sheep, mouse and the already mentioned rabbit milk has been recently established (Paleyanda et al., 1997; Niemann et al., 1999; Chen et al., 2002; Chrenek et al., 2005). In comparison with large domestic animals, rabbit is a promising alternative thanks to its short reproduction cycle and easy milking of the protein-rich milk.

On the other hand, there are many factors influencing the efficiency of transgenic rabbit production. One of the most important obstacles is the low rate of transgene incorporation into the genome of the microinjected embryos and the stability of the transgene transmission to next generations. Our previous studies (Chrenek et al., 2005; 2007) confirmed vertical transfer of the *hFVIII* transgene through two generations (F2 and F3) by PCR, Southern blotting, real-time PCR and Western blotting. However, these methods are not capable of determining the number of transgene copies in the genome or their positions in particular chromosomes.

In the present study we introduced fluorescence *in situ* hybridization coupled with a tyramide amplification step (FISH-TSA) using a short cDNA probe in order to identify chromosomal position(s) of the *hFVIII* gene in transgenic rabbit genomes of F2 and F3 generation.

Received June 25, 2008. Accepted August 1, 2008.

This work was supported by the Slovak Research and Development Agency (Contract No.: LPP-0126-06) and by the Ministry of Education, Youth and Sports of the Czech Republic (Grant No.: MSM0021620858).

Corresponding author: Vladimir Krylov, Charles University in Prague, Faculty of Science, Department of Cell Biology, Viničná 7, 128 43, Prague 2, Czech Republic.

Phone: +420 221 951 773; Fax: +420 221 951 758; e-mail: vkrylov@natur.cuni.cz

Abbreviations: FISH-TSA – fluorescence *in situ* hybridization coupled with tyramide signal amplification step, hFVIII – human coagulation factor VIII.

Material and Methods

Animals

Transgenic founders with the *WAP-hFVIII* gene were produced as described by Chrenek et al. (2005). In our experiments we used a transgenic male parent (F2 generation) and two offspring (F3 generations). Randomly selected non-transgenic rabbits of the same breed were used as a control.

Chromosome sample preparations

Fixed cell suspension for preparation of metaphase spreads was obtained from venous blood samples according to Parkányi et al. (2004). Dropping and slide pretreatment prior to FISH hybridization was done according to Krylov et al. (2007).

Sequencing of the rabbit FVIII gene

Primers for RT-PCR of the rabbit *FVIII* gene were designed on the basis of ENSOCUT00000000145, fragmented sequence obtained from the Ensembl database. Seven cDNA amplificates, covering 6667 bp of a partial coding sequence, were cloned using a TOPO XL cloning kit (Invitrogen, Carlsbad, CA). The sequencing was done with AGOWA (Berlin, Germany).

Probe preparation and FISH-TSA

The *hFVIII* transgene probe was prepared by restricting the WAP-hFVIII vector with *Kpn*I. The appropriate DNA fragment (1250 bp), free of any repetitive sequences, was isolated from the agarose gel and then purified on a column of Gel extraction kit (Qiagen, Fargo, ND). Labelling of 1 μg of the template DNA was performed by the random primer technique (DecaLabel DNA labelling kit, Fermentas, Vilnius, Lithuania) with Dig-11-dUTP nucleotide (Roche, Basel, Switzerland), according to the manufacturer's manual. Purification of the reaction mixture was finished on a column of Gel extraction kit (Qiagen). Template DNA for probe preparation of the rabbit orthologous gene was amplified by PCR reaction, using the sequenced cDNA clone and the following primers: 5′ CAAGCCGGCCATATAACATC 3′ and 5′ TGGCATATTTGGGGATCCTA 3′. Purification of the PCR amplificate (1310 bp) and its labelling was conducted according to the same protocol as for the human transgene probe.

FISH-TSA was carried out according to Krylov et al. (2007). In brief, the chromosomes were simultaneously denatured under a cover glass for 5 min at 70 °C with approx. 30 ng of the appropriate probe in simple hybridization buffer (50% formamide, 2x SSC – 300 mM NaCl, 30 mM sodium citrate, pH 7.0) and incubated overnight (12–16 h) at 37 °C. Posthybridization washing was performed three times in 50% formamide in 2x SSC at 42 °C, three times in 2x SSC at room temperature (RT) and one time in 1x TNT buffer (100 mM Tris/HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) at RT, each for 5 min. Visualization of the hybridized probe was accomplished by antidigoxigenin-POD, Fab fragments antibody (Roche). Amplification of the FISH signals was carried out with a TSA-tetramethylrhodamine kit (NEN, Life Science, Boston, MA) according to the manufacturer's manual. The slides were then mounted in Mowiol-DAPI (4', 6'-diamino-2-phenylindole, 500 ng/ml) and observed under a fluorescence microscope using the appropriate filters.

Images of the FISH signals and metaphase spreads were taken separately and merged in pseudocolours. Labelled chromosomes were identified using the p/q arm ratio and their relative size, with the total length of the largest chromosome 1 as an internal standard. The ratios were calculated from DAPI-stained metaphases and were identical to those obtained from an ideogram published by Hayes et al. (2002). Image merging and chromosome measuring was performed using ACC (Sofo, Brno, Czech Republic) software for image analysis.

Results

Identification of DAPI-stained rabbit chromosomes

Individual DAPI-stained chromosomes were recognized by calculating the p/q arm ratio and the ratio of the total length of the examined chromosome and of the largest chromosome 1 (Table 1). Values gained from the R-banded rabbit ideogram (Hayes et al., 2002) served as the standard. The DAPI-stained rabbit karyotype with all the identified chromosomes is shown in Fig. 1a.

Table 1. Identification of all rabbit chromosomes on the basis of p/q arm ratio and the relative length with chromosome 1 as an internal standard.

chromosome	p/q arm ratio	chromosome relative length[a]
1	0.84	1.00
2	0.87	0.88
3	0.63	0.72
4	0.58	0.51
5	0.87	0.34
6	0.98	0.29
7	0.43	0.80
8	0.48	0.56
9	0.73	0.53
10	0.64	0.40
11	0.56	0.36
12	0.17	0.69
13	0.25	0.71
14	0.20	0.71
15	0.25	0.59
16	0.40	0.45
17	0.33	0.48
18	0	0.42
19	0	0.30
20	0.13	0.20
21	0.23	0.21
X	0.50	0.55
Y	0.40	0.23

[a] ratio of the length of the examined chromosome and the length of chromosome 1

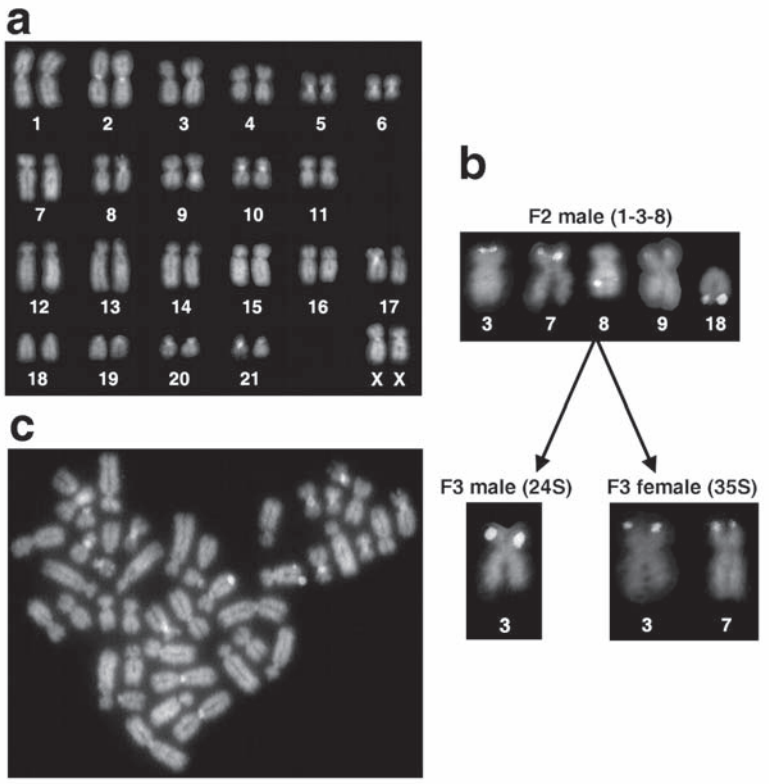

Fig. 1. FISH-TSA analysis of *FVIII* genes. (a) DAPI-stained rabbit karyotype of a non-transgenic female. (b) Localization of the human *FVIII* transgene into rabbit chromosomes of F2 male and two F3 offspring. (c) Localization of the rabbit orthologous *FVIII* gene into the q arm terminus of chromosomes X in a non-transgenic female.

FISH-TSA analysis

FISH-TSA was performed in order to identify the chromosomal position of the human coagulation factor VIII (hFVIII) in an F2 male transgenic rabbit (animal 1-3-8) and two offspring (F3 animals – ♂24s and ♀35s). In the F2 rabbit, the hybridization of the hFVIII probe resulted in labelling of chromosomes 3p, 7p, 8q, 9p and 18q. Animal 24s revealed signal position in p arms of chromosome 3. F3 rabbit 35s had two chromosomes targeted, 3p and 7p (Fig. 1b). The FISH-TSA analysis performed with wild-type animals gave no specific labelling (not shown). To exclude the possibility that the probe labelled the rabbit's orthologous gene, we sequenced it and made the FISH-TSA analysis. The coding sequence was uploaded and published in the GenBank database under accession number EU447260. Nucleotide alignment of human and rabbit cDNA sequences revealed 86% identity and 21 gaps. The corresponding protein structure shows 78% identities and 86% positives when compared to its human counterpart. The rabbit cDNA probe (1310 bp) localized the gene into the q arm terminus of the X chromosome (Fig. 1c).

Discussion

The efficiency of transgenic techniques is determined by the number of integrated transgene copies and their vertical transmission to next generations. In contrast to standard molecular methods such as PCR, real-time PCR, or Southern and Western blotting, only physical mapping of a transgene to the chromosomes by *in situ* hybridization can unfailingly confirm these two parameters. Single-copy gene mapping in rabbit has been based on localizing the long (tens to hundreds kbp) bacterial artificial chromosomes (BACs) bearing the gene of interest by fluorescence *in situ* hybridization (FISH) (Rogel-Gaillard et al., 2001). Propagation and utilization of the BAC library for physical mapping poses many obstacles, such as the loss of once produced bacterial clone due to instability of large DNA constructs, or mapping of surrounding genes and non-coding areas included in a particular BAC clone. Successful mapping of transgenes depends on the sufficient length of the used DNA vector or the respective transgene sequence. The major obstacle, low sensitivity of FISH, was overcome by Bobrow et al. (1992). They introduced a strategy called catalysed reporter deposition (CARD). The FISH signal is amplified by horseradish peroxidase-catalysed deposition of tyramide free radicals in the vicinity of the enzyme. Increasing the sensitivity by tyramide free radical deposition allows for performing the FISH analysis with short probes (from several hundreds bp) (Speel et al., 1997; Schriml et al., 1999). Krylov et al. (2003) simplified this technique and mapped the first amphibian single-copy gene for Src tyrosine kinase (*src-1*) into *Xenopus laevis* chromosomes. The cDNA sequence was used as a probe. Thanks to the absence of repetitive elements, competitor DNA such as salmon

sperm DNA, Cot-1, etc. could be avoided in the hybridization mixture without any negative effect on the signal/background ratio. Tlapakova et al. (2005) showed the high accuracy of this technique in mapping similar sequences of paralogous *MDH2a* and *b* genes in the same species.

In this study we used FISH-TSA for mapping the human coagulation factor VIII transgene in rabbit chromosomes. Identification of targeted chromosomes in DAPI-stained metaphase spreads was done by calculating the p/q arm ratio and the relative length, with chromosome No. 1 as an internal standard. This approach simplifies the analysis and allows the evaluation of FISH-TSA signals without the chromosomal banding procedure. Phillips and Reed (2000) identified all 25 chromosomal pairs in *Danio rerio* on the basis of size and centromere ratios in CMA-3-stained metaphases. Tymowska (1973) and Krylov et al. (2007) used p/q arm ratios for the assessment of Giemsa- and DAPI-stained chromosomes of *Xenopus tropicalis*, respectively. Chromosomal localization of the human *FVIII* transgene in rabbits of F2 and F3 generation clearly showed the vertical separation of individual loci. FISH-TSA performed with a probe for the orthologous rabbit *FVIII* gene revealed terminal position on the q arms of chromosome X. In human, the gene is localized in a similar region (Xq28). Korstanje et al. (1999) used reciprocal chromosome painting between the human and the rabbit. The paint for the human chromosome X was found to hybridize to the rabbit X orthologue only. This finding together with current gene mapping results indicates strong X chromosome conservation between both species.

References

Bobrow, M. N., Litt, G. J., Shaughnessy, K. J., Mayer, P. C., Conlon, J. (1992) The use of catalyzed reporter deposition as a means of signal amplification in a variety of formats. *J. Immunol. Methods* **150**, 145-149.

Bozse, Z., Hiripi, L., Carnwath, J. W., Nieman, H. (2003) The transgenic rabbit as a model for human diseases and as a source of biologically active recombinant proteins. *Transgenic Res.* **12**, 541-553.

Chen, C. M., Wang, C. H., Wu, S. C., Lin, C. C., Lin, S. H., Cheng, W. T. K. (2002) Temporal and spatial expression of biologically active human factor VIII in the milk of transgenic mice driven by mammary-specific bovine α-lactalbumin regulation sequences. *Transgenic Res.* **11**, 257-268.

Chrenek, P., Vasicek, D., Makarevich, A. V., Jurcik, R., Suvegova, K., Parkanyi, V., Bauer, M., Rafay, J., Batorova, A., Paleyanda, R. K. (2005) Increased transgene integration efficiency upon microinjection of DNA into both pronuclei of rabbit embryos. *Transgenic Res.* **14**, 417-428.

Chrenek, P., Ryban, L., Vetr, H., Makarevich, A. V., Uhrin, P., Paleyanda, R. K., Binder, B. R. (2007) Expression of recombinant human factor VIII in milk of several generations of transgenic rabbits. *Transgenic Res.* **16**, 353-361.

Fan, J., Watanabe, T. (2003) Transgenic rabbits as therapeutic protein bioreactors and human disease models. *Pharmacol. Ther.* **99**, 261-282.

Hayes, H., Rogel-Gaillard, C., Zijlstra, C., De Haan, N. A., Urien, C., Bourgeaux, N., Bertaud, M., Bosma, A. A. (2002) Establishment of an R-banded rabbit karyotype nomenclature by FISH localization of 23 chromosome-specific genes on both G- and R-banded chromosomes. *Cytogenet. Genome Res.* **98**, 199-205.

Korstanje, R., O'Brien, P. C., Yang, F., Rens, W., Bosma, A. A., van Lith, H. A., van Zutphen, L. F., Ferguson-Smith, M. A. (1999) Complete homology maps of the rabbit (*Oryctolagus cuniculus*) and human by reciprocal chromosome painting. *Cytogenet. Cell Genet.* **86**, 317-322.

Krylov, V., Macha, J., Tlapakova, T., Takac, M., Jonak, J. (2003) The c-*src*1 gene visualized by *in situ* hybridization on *Xenopus laevis* chromosomes. *Cytogenet. Genome Res.* **103**, 169-172.

Krylov, V., Tlapakova, T., Macha, J. (2007) Localization of the single copy gene *Mdh2* on *Xenopus tropicalis* chromosomes by FISH-TSA. *Cytogenet. Genome Res.* **116**, 110-112.

Niemann, H., Halter, R., Carnwath, J. W., Herrmann, D., Lemme, E., Paul, D. (1999) Expression of human blood clotting factor VIII in the mammary gland of transgenic sheep. *Transgenic Res.* **8**, 137-149.

Paleyanda, R. K., Velander, W. H., Lee, T. K., Scandella, D. H., Gwazdauskas, F. G., Knight, J. W., Hoiyer, L. W., Drohan, W. N., Lubon, H. (1997) Transgenic pigs produce functional human factor VIII in milk. *Nat. Biotechnol.* **15**, 971-975.

Parkányi, V., Chrenek, P., Rafay, J., Süvegová, K., Jurcík, R., Makarevich, A. V., Pivko, J., Hetényi, L., Paleyanda, R. K. (2004) Aneuploidy in the transgenic rabbit. *Folia Biol. (Praha)* **50**, 194-199.

Phillips, R. B., Reed, K. M. (2000) Localization of repetitive DNAs to zebrafish (*Danio rerio*) chromosomes by fluorescence *in situ* hybridization (FISH). *Chromosome Res.* **8**, 27-35.

Rogel-Gaillard, C., Piumi, F., Billault, A., Bourgeaux, N., Save, J. C., Urien, C., Salmon, J., Chardon, P. (2001) Construction of a rabbit bacterial artificial chromosome (BAC) library: application to the mapping of the major histocompatibility complex to position 12q.1.1. *Mamm. Genome* **12**, 253-255.

Schriml, L. M., Padilla-Nash, H. M., Coleman, A., Moen, P., Nash, W. G., Menninger, J., Jones, G., Ried, T., Dean, M. (1999) Tyramide signal amplification (TSA)-FISH applied to mapping PCR-labeled probes less than 1 kb in size. *Biotechniques* **27**, 608-613.

Speel, E. J., Ramaekers, F. C., Hopman, A. H. (1997) Sensitive multicolor fluorescence *in situ* hybridization using catalyzed reporter deposition (CARD) amplification. *J. Histochem. Cytochem.* **45**, 1439-1446.

Tlapakova, T., Krylov, V., Macha, J. (2005) Localization, structure and polymorphism of two paralogous *Xenopus laevis* mitochondrial malate dehydrogenase genes. *Chromosome Res.* **13**, 699-706.

Tymowska, J. (1973) Karyotype analysis of *Xenopus tropicalis* Gray, Pipidae. *Cytogenet. Cell Genet.* **12**, 297-304.