Expression of the Transgene is Consistently Inherited to High Numbers of Generations and Independent on Its Source

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ABSTRACT

Most studies on transgenic bioreactors have focused on expression levels of interest genes. In this study we examined whether transgenic bioreactors would inherit expression level of the transgene to long-term generations independently of transgene sources. We employed three transgenic mice, which were separately reported, carrying different transgenes and copy numbers, 27 kb of *hLF* and 22 kb of *hIL-10* genomic sequences, and 1.3 kb of *hTPO* cDNA, respectively. Three females of the transgenic lineages crossbred with a wild-type male up to 20 generations to test transgenic frequencies of their progenies and to determine expression levels of the transgenes. Ultimately, transmission rates of *hLF*, *hIL-10*, and *hTPO* were 64.3±7.0, 59.3±9.8, and 56.1±9.7, respectively, appeared following Mendelian pattern of inheritance. Notably, we found that levels of expressions of hLF, hIL-10, and hTPO in milk were sustained to high numbers of generations. No transgene silencing of expression was observed in every generations of all transgenic mice. In conclusion, we suggest that once established animal bioreactors could consistently transmit the transgene to continual generations, without loss of expressional activity, independently of transgene sources.

(Key words : Human lactoferrin, Human TPO, Human IL-10, Transmission)

INTRODUCTION

The transgenic animal technology has been used as a powerful tool to produce human proteins into milk, which could offer a renewable source of commercially important proteins (Rudolph, 1999). Since the first recombinant human protein was expressed in the milk of transgenic mouse (Gordon *et al.*, 1992), much of efforts have been performed to generate transgenic animals producing human proteins at commercially feasible levels over 1 mg/ml (Gordon *et al.*, 1992; Houdebine 2000). While highlighted to be successful in generation of transgenic animal, ultimately, all transgenic animals generated did not express sufficiently target genes, accounting for positional effect of an integrated transgene into host chromosome.

For past decade, we have isolated and characterized milk protein promoters to find available regulatory elements that control mammary gland- and lactating-specific gene expression, from various animals, including mouse (Lee *et al.*, 1998), rat (Lee *et al.*, 1996), cow (Kim *et al.*, 1999; Oh *et al.*, 1999), and goat (Lee *et al.*, 2000). Among those, the isolated promoters from bovine and caprine β-casein genes have been extensively employed to generate transgenic mice and led to high level expression of various therapeutic proteins, lysozyme (Lee *et al.*, 1998), *hLF* (Han *et al.*, 2005; Kim *et al.*, 1997; Kim *et al.*, 1999), *hTPO* (Sohn *et al.*, 1999), growth hormone (Lee *et al.*, 1996; Choi *et al.*, 1998; Oh *et al.*, 1999), human interleukin-10 (Sohn *et al.*, 2003), and granulocyte colony stimulating factor (Ko *et al.*, 2000) into their milk. Eventually, we have generated transgenic livestock animals, Korean native goat (Ko *et al.*, 2000; Lee *et al.*, 2000), and cow (Han *et al.*, 2005).

Although transgenes into germ-line transmitted were generally known to be highly stable on integration site to high number of generations of transgenic animals (Aigner *et al.*, 1999; Markaki *et al.*, 2007), variegation of expression level of transmitted transgene has been frequently reported by reasons of genetic or epigenetic modification of transgene dependently of its integration sites (Dobie *et al.*, 1996; Dobie *et al.*, 1997; Migliaccio *et al.*, 2000; Opsahl *et al.*, 2003). These reports have led to

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us to inquire whether expression level of transgene could be sustained continually to long term generations independently of its source. We partially reported that expression level of a single copy integrated transgene was inherited through germline (Zheng *et al.*, 2003; Zheng *et al.*, 2004). In this paper, we performed propagation of selected transgenic mice carrying different sources of transgenes *hLF*, *TPO*, and *hIL-10* and then analyzed those expression levels up to 20 generations.

MATERIALS AND METHODS

Transgenic Mice used to Test Pattern of Transgene Inheritance

Previously we reported separately generations of transgenic mice that was microinjected of transgene into the male pronuclei of the BCF1 (C57BL/6×CBA) mouse embryos expressing *hLF* (Kim *et al.*, 1999), *hIL-10* (Sohn *et al.*, 2003), and *hTPO* (Sohn *et al.*, 1999) into milk. Briefly, the mammary-expression vectors consist of different coding regions, 27 kb of genomic *hLF* DNA, 22 kb of genomic *hIL-10* DNA sequences, and 1.3 kb of *hTPO* cDNA sequences, respectively, in conjunction with identical 10 kb promoter region of bovine β-casein gene. In this study, we used each one lineage of three independent transgenic mice, as shown in Table 1, which were at highest level of transgene expression among various lineages (Kim *et al.*, 1999; Sohn et al., 1999; Sohn *et al.*, 2003).

Propagations and Genotyping of Three Independent Transgenic Mice

The scheme of propagation of transgenic mouse was illustrated in Fig. 1. Three transgenic mice females were crossbred with a wild type male (C57BLxDBA strain). All progenies were genotyped using PCR analysis with a transgene-specific primer, followed previous report (Kim *et al.*, 1999; Sohn *et al.*, 1999; Sohn *et al.*, 2003). Propagation and genotyping of every transgenic mouse were repeated up to 20 generations (F20) to exam pattern of transgene inheritance.

Analysis of Expression Level of Transgene by ELISA

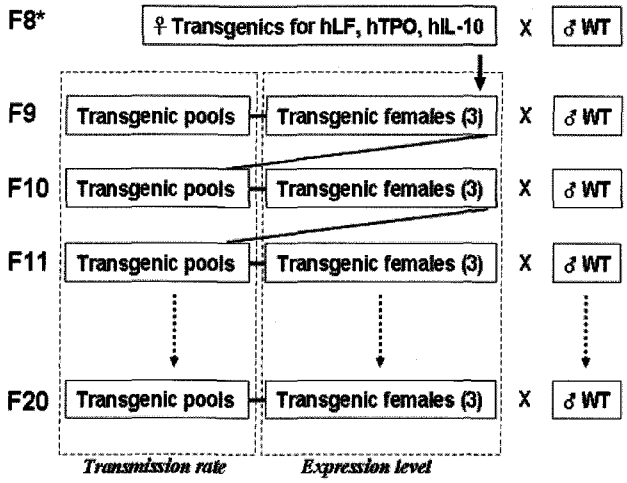

Fig. 1. Diagram of propagation of three independent transgenic mice. *Generations, which are initially used, are differed between transgenes. See detail in materials and methods.

The expression level of transgene was determined by ELISA. Three females, which were randomly chosen among transgenic progenies identified as transgenic mice by genotyping analysis, were crossbred with a wild type male, and then allowed to nurse all progenies after parturition. Milk was collected from transgenic females of 10-day lactation. The process for collecting and sampling of milk in detail were described in previous studies (Kim *et al.*, 1999; Sohn *et al.*, 1999; Sohn *et al.*, 2003). The concentrations of hLF, hTPO, and hIL-10 were separately measured using an ELISA kit following instructions of manufactures (Calbiochem, USA; R & D system; Phar-Mingen, respectively).

RESULTS

The characteristics of transgene constructs for *hLF*, *hTPO* and *hIL-10* expressions, and their transgenic mice were well demonstrated in previous studies (Kim *et al.*, 1999; Sohn *et al.*, 1999; Sohn *et al.*, 2003). The *hTPO* construct consisted of 10 kb of bovine β-casein promoter, 1.3 kb of *hTPO* cDNA including poly (A) signal sequence of bovine growth hormone gene (Sohn *et al.*, 1999). Am-

Table 1. Profile of transgenic mice used to test pattern of transgene inheritance

Transgene	Transgenic line	Transgene source	Copy number	Expression level (mg/ml)	Reference
hLF	23	27 kb of gDNA	4	6.6	(Kim *et al.*, 1999)
hTPO	15	1 kb of cDNA	1	1.5	(Sohn *et al.*, 1999)
hIL-10	6	12 kb of gDNA	1	1.6	(Sohn *et al.*, 2003)

ong transgenic founders, a lineage 15, which has been carrying a single copy of it, was shown to stably transmit *hTPO* transgene, and its expression to F 10 (Zheng *et al.*, 2003). In this study, we examined additionally pattern of *hTPO* transgene inheritance up to F20. As presented in Table 2, transgenic rates were variable between generations, showing 46.7% of F18, whereas 78.6% of F11. However, the mean rate descending to progenies of *hTPO* transgene trough F20 was 56.1±1.0%, showing Mendelian pattern of inheritance. The expression level, which was comparable to a founder's, was shown to be consistent between siblings and generations, indicating that expression of a protein cDNA sequence-construct is transmitted constantly level to long-term generations.

Recently, we reported that *hIL-10* genomic DNA construct whose a single copy is integrated into a chromosome, were transmitted and actively expressed through F15 (Zheng *et al.*, 2004). In addition, from F15 to F20 we confirmed that the lineage expressing *hIL-10* has perpetuated the transgene integrity and its expression (Table 2 and 3), although variation of expression levels between siblings was observed (F16).

Finally, we investigated inheritance of multiple copies of 37 kb of a transgene, consisting of 27 kb of *hLF* genomic DNA and 10 kb of bovine β-casein promoter (Table 1). We employed a lineage 23 of transgenic mouse, of which the founder expressed hLF at level of 6.6 mg/ml (Table 1). Propagation, test that was performed from F8 through F20, showed that *hLF* construct was stably transmitted, comparable to constructs for hTPO and hLF expressions (64.3±7.0). The expression level varied between siblings, thus appearing to be lower than founder's. However, we could observe many of mice expressing hLF at maximum levels, which were over 6 mg/ml, on some generations. In addition, discrepancy between individual's expression levels of same generation was not more than two fold, likely to reflect that different physiological context causes different expression levels between siblings. We suggest that 4.0±0.6 mg/ml of the expression level might be the potential competence for hLF expression of a lineage 23. Taken together, we suggest that the foreign DNA, which is exogenously integrated and transmitted to germline, would be preserved in its integrity and expressional activity for long generations, and to be independent on its source.

DISCUSSION

It is clear that animal bioreactor using mammary gland of dairy animals allow massive production of commercially important proteins into milk. And, it is believed that transgenic bioreactor animal would inherit transgene and its expression level to next generations. However, so far, experimental substantiation has not

Table 2. Stable inheritance of three transgenes

Transgene	*hLF*			*hTPO*			*hIL-10*		
Generation	No. of offsprings born	No. of transgenic mice	Transgenic rate (%)	No. of offsprings born	No. of transgenic mice	Transgenic rate (%)	No. of offsprings born	No. of transgenic mice	Transgenic rate (%)
F9	24	14	58.3	-	-	-	-	-	-
F10	23	18	78.3	-	-	-	-	-	-
F11	21	13	61.9	28	22	78.6	-	-	-
F12	21	12	57.1	25	12	48	-	-	-
F13	23	14	60.9	35	23	65.7	-	-	-
F14	25	16	64	27	15	55.6	-	-	-
F15	30	19	63.3	24	13	54.2	-	-	-
F16	19	11	57.9	24	14	58.3	18	13	72.2
F17	21	13	61.9	26	13	50	20	9	45
F18	22	17	77.3	30	14	46.7	20	12	60
F19	22	15	68.2	28	15	53.6	21	12	57.1
F20	21	13	61.9	24	12	50	21	13	61.9
Total	272	175	64.3±7.0	271	153	56.1±1.0	100	59	59.3±1.0

Table 3. Transgene expression patterns of inheritance

Transgene	*hLF*		*hTPO*		*hIL-10*	
Generation	Range (mg/ml)	Expression level (mg/ml)	Range (mg/ml)	Expression level (mg/ml)	Range (mg/ml)	Expression level (mg/ml)
Founder	-	6.6*	-		-	-
F9	2.832~4.021	3.2	-	-	-	-
F10	2.462~4.021	3.1	0.603~1.505	1.1**	-	-
F11	3.520~6.122	4.6	0.848~1.370	1.1	-	-
F12	2.640~3.495	3.1	0.848~1.378	1.1	-	-
F13	3.258~6.122	4.5	0.626~1.426	1	-	-
F14	3.258~5.211	4.2	1.059~1.159	1.1	4.2~10.2	6.8***
F15	3.520~6.122	4.6	1.054~1.256	1.1	5.510~8.237	6.8
F16	3.485~4.021	3.8	0.987~1.189	1.1	2.489~9.262	6.3
F17	3.057~4.021	3.4	1.059~1.159	1.1	4.211~7.121	5.7
F18	3.037~5.863	4.1	1.059~1.111	1.1	4.045~7.122	5.6
F19	3.037~6.122	4.4	0.987~1.159	1.1	4.211~8.237	6.4
F20	3.258~6.122	4.5	0.987~1.122	1.1	5.510~7.121	6.1
Total	2.5~6.1	4.0±0.6	0.6~1.5	1.1±0.03	2.5~12.0	6.2±0.1

* (Kim *et al.*, 1999); ** (Zheng *et al.*, 2003); *** (Zheng *et al.*, 2004).

been presented in our knowledge, although for biological purpose and non-mammalian the few were reported (Aigner *et al.*, 1999; Wu *et al.*, 2005; Markaki et al., 2007). To substantiate that expression of transgene is inherited stably, we examined expression pattern of selected transgenes up to 20 generations. To reduce genetic and epigenetic variation between generations and transgene sources, we employed independent three transgenic mice carrying different transgene sources, and genotyped more than 18 progenies obtained from those females. The consequence clearly demonstrated that germ-line transmitted transgenes is stably inherited to high numbers of generations.

In our original studies, we used one founder mouse to analyze expression levels of multiple copies of *hLF*, and of single copy of *hTPO*, and *hIL-10*. Interestingly, in this paper, while *hTPO*-cDNA was shown to be expressed at relatively constant level between siblings, *hLF* and *hIL-10* genomic DNA appeared to be variable levels between siblings. Since a *hIL-10* construct, which was identified as one copy integration (Table 1), was stably transmitted (Table 2), it is likely that genetic modification of transgene, such as its elimination from host chromosome (Migliaccio *et al.*, 2000), did not cause the variation to occur. It could be postulated that a distinctive difference of milk productivity between mothers, by different physiological contexts, lead to different transcriptional potential of whole milk protein genes, including a foreign DNA of 10 kb of bovine β-casein promoter contained available responsive sequences to lactogenic signal. In contrast, the *hLF* transgenic mouse was confirmed to carry four copies of its constructs into chromosome, maybe increased the possible chance of heterochromatic condensing of multiple transgenes (Dobie *et al.*, 1997). However, *hLF* transgene silencing of expression was not observed. This allowed us to presume that heterochromatic-condensing of multiple integrated *hLF* is not a consequence of variable expression between siblings. Alternatively, different physiological competence to produce milk proteins and/or the *hLF* construct-specific mosaic expression pattern by position effect of heterozygous transgenes would be possible explanation. Notably, the variation of expression level in transgenic mice examined in this paper was not inherited through high number of generations (F20), giving further emphasis to us that animal bioreactor is useful a biotechnological system.

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