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# Efficient integration and expression of β-casein hybrid genes in transgenic mice as animal bioreactor models

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There is a considerable interest in the production of recombinant proteins from the milk of transgenic animals, as an alternative to large-scale mammalian cell culture system. A number of milk protein genes have been isolated and shown to function efficiently in the mammary gland of transgenic mice. So far, several heterologous proteins have been expressed in the milk of many transgenic animals using regulatory sequences of various milk protein genes. In particular, several groups have reported high level expression of hybrid genes in the milk of transgenic mice. Actually, this approach is now in the process of industrial application for a large scale production of pharmaceutically active proteins from transgenic livestock.

To apply this technology to farm animals, a fiedelity of transgene expression has to be precisely evaluated in transgenic mouse models.

### *1. An Efficient Expression of Human Growth Hormone (hGH) in the Milk of Transgenic Mice Using Rat β-Casein/hGH Fusion Genes*

Firstly, hGH reporter gene expression driven by the rat β-casein promoter was studied in transgenic mice. The rat β-casein gene encodes the principal murine casein and is abundantly expressed during mammary gland development. In spite of its abundance in the milk, intact and hybrid genes of the rat β-casein gene was inefficiently expressed in transgenic mice. The expression efficiency of hybrid genes does not absolutely depend on the milk protein gene promoter. It is well known that genomic sequences generally show a better performance than their cDNAs when used in an expression construct because introns have some regulatory functions on their expression. The selection of a target gene and the architecture of the hybrid gene could be then crucial for the efficient expression of the construct. In the present study, we examined whether the rat β-casein promoter could direct an efficient production of hGH in the milk of transgenic mice when it was combined with 3' flanking sequences of hGH gene itself (pBCN1GH) or the rat β-casein gene (pBCN2GH).

We observed approximately 15% efficiency in obtaining transgenic mice in terms of the percentage of mice screened. Six and 3 transgenic founder mice were obtained from the pBCN1GH and pBCN2GH constructs, respectively. Three transgenic lines were established from each vector.

Several G1 transgenic females from each line were milked on day 11 of lactation. More than 19μg/mℓ of hGH was detected in the milk of BCN1GH transgenic lines (11, 25, and 28), while less than 2μg/mℓ of hGH was present in the milk of BCN2GH lines (1, 7, and 9). The highest level of hGH was 5500±620μg/mℓ produced in line 11 (Table 1).

Table 1. Concentration of hGH in the milk and blood of transgenic mice

| Transgenic line | Copy[a] number | hGH[b](μg/mℓ) in Milk | Blood | |
|---|---|---|---|---|
| | | | lactating | cyclic |
| BCN1GH | | | | |
| 11 | 20 | 5500±620 | 0.556 | 0.02 |
| 25 | 10 | 19±4 | 0.005 | <0.001 |
| 28 | 20 | 30 | 0.027 | 0.002 |
| BCN2GH | | | | |
| 1 | 2 | 0.7 | 0.002 | <0.001 |
| 7 | 30 | 2.0±0.4 | 0.002 | <0.001 |
| 9 | 30 | 0.7 | 0.003 | <0.001 |
| Non-transgenic control | | <0.1[c] | <0.001 | <0.001 |

[a]The transgene copy numbers were approximately estimated from Southern blot analysis.

[b]The values are means(±s.e.m.)of hGH concentrations determined in more than two mice from each line by RIA kit which detection limit is 0.001μg/mℓ. S.e.m. was represented when three mice were analyzed.

[c]The detection limit for milk was 0.1μg/mℓ because of its dilution to 1/100 to decrease non-specific binding.

hGH concentration in blood was also measured in cyclic and lactating mice (Table 1). In all lactating transgenic lines, variable amounts of hGH were detected in the blood and their levels were higher than those of cyclic mice. For instance, in the line 11, blood hGH level of virgin mouse was 0.02㎍/㎖ and this value increased to 0.556㎍/㎖ by day 11 of lactation.

In order to investigate the tissue specificity of transgene expression, total RNAs of various organs of lines 7, 11 and 28 were subject to Northern blot analysis (Fig. 1). hGH mRNA was detected in the mammary gland, but not in other tissues of the lines. The hybridization signal from line 11 was much stronger than the other lines. Therefore, according to the Northern blot analysis, hGH was exclusively expressed in the mammary gland of the transgenic mice and the amounts of hGH mRNA were shown to correlate well with the amounts of hGH secreted into the milk (Table 1).

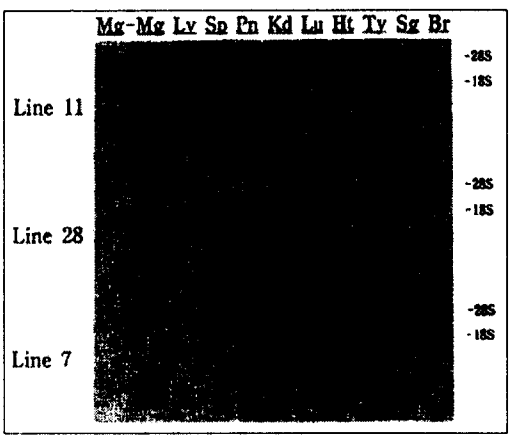


Fig. 1. Tissue-specific hGH expression by Northern blot analysis. Total RNAs (20㎍) from various organs of lines 11, 28 and 7 were applied to a nylon filter and hybridized with the $^{32}$P-labeled 1.1 kb *Pvu*II fragment of hGH gene shown in Fig. 1. The analyzed organs are mammary gland (Mg), liver (Lv), spleen (Sp), pancreas (Pn), kidney (Kd), lung (Lu), heart (Ht), thymus (Ty), salivary gland (Sg), and brain (Br) of transgenic mice and mammary gland of a non-transgenic mouse (Mg-).

Although Northern blot analysis showed that hGH expression was strictly restricted to the mammary gland, sensitive RIA and RT-PCR revealed the production of hGH in several non-mammary organs including thymus, salivary gland, liver, kidney, and lung from some transgenic lines.

Table 2. hGH contents in various tissues of transgenic mice

| Transgenic line | hGH contents (ng/mg protein) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mg[a] | Lv | Sp | Pn | Kd | Lu | Ht | Ty | Sg | Br |
| BCN1GH | | | | | | | | | | |
| 11-1 | 6000 | 4.0 | 0.6 | 1.3 | 5.4 | 2.0 | 1.3 | 2.0 | 16.6 | 1.2 |
| -2 | 6500 | 4.2 | 2.0 | 1.0 | 3.8 | 1.7 | 3.3 | 14.4 | 18.9 | 5.2 |
| 25-1 | 130 | - | - | - | 0.3 | 0.2 | - | 0.8 | 1.4 | - |
| -2 | 90 | - | - | - | - | - | - | 0.6 | 0.3 | - |
| 28-1 | 360 | - | - | - | - | - | - | 3.4 | - | - |
| -2 | 100 | 0.2 | - | - | 2.0 | - | - | 2.7 | 0.4 | - |
| BCN2GH | | | | | | | | | | |
| 1-1 | 2.3 | - | - | - | - | - | - | 0.4 | - | - |
| -2 | 4.0 | - | - | - | - | - | - | 0.4 | - | - |
| 7-1 | 9.4 | - | - | - | - | - | - | 1.1 | - | - |
| -2 | 11.0 | - | - | - | - | - | - | 5.3 | 0.3 | - |
| 9-1 | 2.3 | - | - | - | - | - | - | 7.8 | 0.3 | - |
| -2 | 1.9 | - | - | - | - | - | - | 1.2 | 0.3 | - |

[a]Tissue extracts were prepared from two lactating mice from each transgenic line. Organs are abbreviated by Mg, mammary gland; Lv, liver; Sp, spleen; Pn, pancreas; Kd, kidney; Lu, lung; Ht, heart; Ty, thymus; Sg, salivary gland; Br, brain.

-; < 0.2 ng/mg protein

It has been known that the rat β-casein gene expression is temporally regulated during mammary gland development. To determine whether the rat β-casein/hGH fusion genes also exhibit developmental regulation, slot blot analysis was performed using total RNAs isolated from the mammary gland of lines 11 and 28 at virgin, day 17 of pregnancy, and day 11 of lactation (Fig. 2).

hGH mRNA levels were markedly increased from virgin through pregnancy to midlactation in the two transgenic lines. Moreover, this expression pattern of hGH transgene was similar to that of the endogenous mouse β-casein gene. These results indicate that the rat β-casein/hGH expression can be developmentally regulated in the mammary gland just like the endogenous mouse β-casein gene.

In summary, the rat β-casein promoter could direct an efficient production of hGH in a highly tissue- and stage-specific manner when it was combined with the 3′ sequences of the hGH gene.

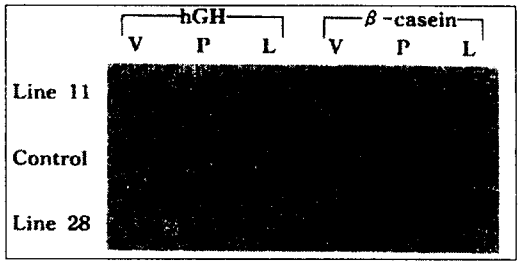


Fig. 2. Developmental regulation of hGH and β-casein expression in the mammary gland of transgenic mice. Total RNAs (5μg) isolated from the mammary gland of lines 11, 28, and non-transgenic control mice at virgin (V), 17-day pregnant (P), or 11-day lactating (L) stage were blotted on a nylon filter and hybridized with either an 1.1 kb hGH fragment shown in Fig. 1 or a 0.8 kb *Sal*I/*Eco*RI fragment of rat β-casein gene containing exon VII.

### *2. Dual tissue-specificity of bGH transgene expression directed by bovine β-casein promoter in transgenic mice*

Here we report a unique regulatin of the hybrid gene expression controlled by bovine, not murine, β-casein promoter in transgenic mice. The proximal promoter sequences of several β-casein genes of different species are well conserved and several common sequence elements have been defined in this region. Despite the sequence similarity in the proximal promoter region of the β-casein genes, prominent differences were found between bovine and murine β-casein genes in hormone responsiveness in vitro and in regulatory patterns during mammary gland development. Therefore it is crucial to define the sequence requirement for the proper regulation of the bovine β-casein gene in transgenic mice and thus we investigated the temporal and spatial regulation of bGH transgene expression controlled by the 1.8 kb of the bovine β-casein promoter in transgenic mice.

Nine transgenic founder mice were identified by PCR analysis. Milk samples could be collected from G0 or G1 female mice of seven transgenic lines. Western blot analysis estimated concentration of bGH in milk samples, which was ranged from undetectable level in lines 3, 8, and 9 up to 1,600 μg/ml in line 6. We then analyzed tissue specificity of bGH transgene expression in the lines 1, 2, 4 and 6, which secreted bGH into their milk. Unexpectedly, bGH transgene was expressed in the lung as well as in the mammary gland, but not in other tissues including liver, spleen, pancreas, kidney, heart, salivary gland, and brain in all the four lines (Fig. 3). Moreover, bGH mRNA level in the lung was considerably high without correlation to that in the mammary gland of each line. To confirm the consistency of bGH transgene expression in the lung, the lines 3, 8, and 9, which showed undetectable levels of bGH in milk, were analyzed. Two of the three lines also showed high level of bGH transgene expression in the lung and especially bGH mRNA level in line 8 was highest in all lines tested. These results demonstrated that bovine β-casein/bGH transgene directs a dual tissue specificity of transgene expression in both mammary gland and lung with different regulatory mechanisms.

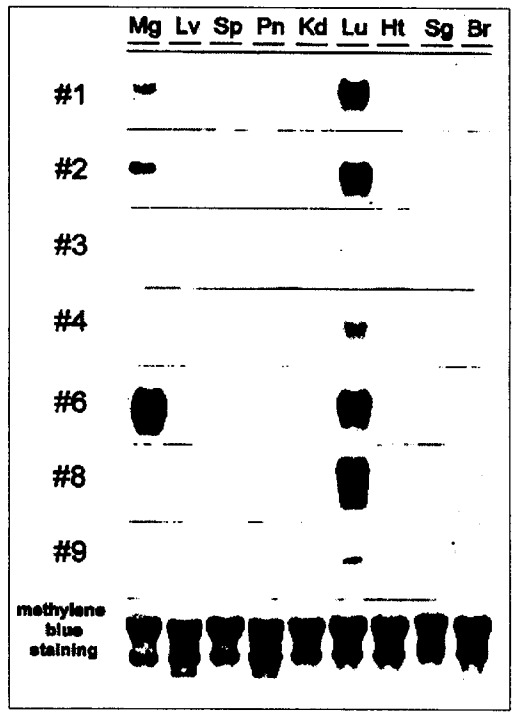


Figure 3. Tissue-specific expression of bGH transgene in transgenic mice. Total RNA samples(30mg) of various tissues of lactating transgenic mice were subjected to Northern blot analysis. Analyzed tissues were mammary gland(Mg), liver(Lv), spleen(Sp), pancreas(Pn), kidney(Kd), lung(Lu), heart(Ht), salivary gland(Sg), and brain(Br). Blots were hybridized to a bGH-specific probe. Transgenic lines were indicated as # numbers. An example of methylene blue stained blot (line 2) was shown below to confirm the integrity and quantity of the purified total RNA.

In addition, to investigate the temporal regulation transgene expression, the expression of bGH transgene and endogenous β-casein gene during mammary gland development was studied in two independent transgenic lines 2 and 6 by Northern blot analysis (Fig. 4). No bGH mRNA signal was detected in both lines until 15-day pregnant stage, and then a strong induction of

bGH expression occurred at lactating mammary glands of both lines (Fig. 4A). In contrast, initial increase of endogenous mouse β-casein gene expression occurred at pregnant stage and peaked at lactating stage. At involution stage, bGH transgene and endogenous β-casein gene showed a similar expression pattern: Their expression was gradually decreased after weaning. These results demonstrated that the 1.8 kb promoter of the bovine β-casein gene could accurately regulate transgene expression in a bovine-specific temporal pattern rather than a murine-specific during the mammary gland development.

The lung-specific expression was also measured at different developmental stages of mammary gland because bGH transgene was specifically expressed in the lung of lactating mice (Fig. 4B). In contrast to the mammary gland expression, bGH mRNA level in the lung was almost constant throughout mammary gland development in both lines.

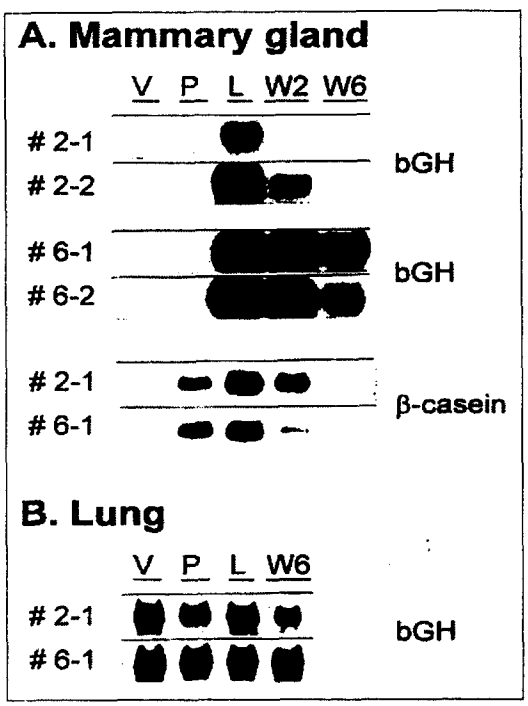


Figure 4. Temporal expression patterns of bGH transgene and endogenous b-casein in the mammary gland and lung during mammary gland development. Total RNAs were isolated from the mammary gland (A) and the lung (B) at different developmental stages of transgenic mice (#2 and #6) and subjected to Northern blot analysis. Different amounts of total RNA were used for detecting the expression of bGH (30mg) and endogenous b-casein (5mg). Transgenic lines were indicated as # numbers. Developmental analysis of bGH expression in the mammary gland of lines 2 and 6 were duplicated with two individuals in each line (A). V, virgin; P, pregnancy; L, lactation; W2, 2-day after weaning; W6, 6-day after weaning.

To precisely determine the cellular type of bGH transgene expression, *in situ* hybridization and immunohistochemical staining were carried out using tissue sections of the mammary gland and lung from a 10-day lactating mouse of line 6 which showed highest level of bGH transgene expression in the mammary gland of transgenic mice. In the mammary gland, hybridization signals probed with antisense bGH riboprobe were presented in most of the cells of the secretary epithelium lining alveoli. An intensive signal for bGH protein was homogeneously detected by immuno- histochemical analysis within the same epithelial cells of alveoli. In the lung, however, the bGH signals were not homogeneous, but rather cell-type specific in mRNA and protein levels. Both assays revealed that the type II pneumocyte was a specific cell type expressing bGH transgene in the lung.

Therefore these results demonstrated that the 1.8 kb promoter of the bovine b-casein gene could directs a highly distinct, dual tissue-specificity of bGH expression in both mammary gland and lung with different regulatory mechanisms, although the promoter fragment was sufficient for the accurate temporal regulation of the reporter gene expression in the mammary gland.

In conclusion, the rat and bovine β-casein gene promoters could direct high level expression of growth hormone in the milk of transgenic mice. However the two promoters differently regulated GH transgenes in temporal- and spatial-specific expression. Therefore it is crucial to use the host-specific gene promoter for the accurate regulaton of the transgenene expression in transgenic animals.

## REFERENCES

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