Accurate Spatial and Temporal Transgene Expression Driven by a 3.8-Kilobase Promoter of the Bovine β-Casein Gene in the Lactating Mouse Mammary Gland

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ABSTRACT The spatial, temporal, and hormonal pattern of expression of the β-casein gene is highly regulated and confined to the epithelial cells of the lactating mammary gland. Previous studies have shown that 1.7 kb of the bovine β-casein promoter were able to drive cell-specific and hormone-dependent expression to a mouse mammary cell line but failed to induce accurate expression to the mammary gland of transgenic mice. We investigated here the ability of 3.8 kb of the bovine β-casein gene promoter to drive the expression of the human growth hormone (hGH) gene in transgenic mice. A Northern blot analysis using total RNA obtained from different tissues of lactating and nonlactating females revealed the presence of hGH mRNA only in the mammary gland of lactating females. hGH mRNA was not detectable in the mammary gland of virgin females or males. A developmental analysis showed that hGH mRNA only peaked on parturition, resembling more closely the bovine β-casein temporal expression pattern rather than the murine. In situ hybridization studies performed on mammary gland sections showed that the cellular pattern of hGH expression was homogeneous in all lobules from heterozygous and homozygous transgenic mice. Silver grain counts on the tissue sections highly correlated with the hGH contents in the milk determined by radioimmunoassay (r = 0.996). Thus 3.8 kb of the bovine β-casein promoter direct a high-level expression of a reporter gene to the lactating mammary gland of transgenic mice in a tissuespecific and developmentally regulated manner. Mol. Reprod. Dev. 49:236–245, 1998. © 1998 Wiley-Liss, Inc.

Key Words: casein genes; transgenic mice; gene expression; milk; human growth hormone

INTRODUCTION Spatial, temporal, and hormonal regulation of mammalian genes is the result of precise interactions that occur between cis-acting elements present in the vicinities of the transcriptional unit of a gene and trans-acting factors capable of recognizing those elements in a highly specific manner. The calcium sensitive casein gene family constitutes an excellent example to study these types of interactions because each of its members displays a highly restricted pattern of expression limited to the epithelial cells of the lactating mammary gland (for a review, see Mercier and Villette, 1993). The onset of expression during the gestation and lactation period is also typical for each of these genes, following an exclusive temporal pattern (Goodman and Schambacher, 1991; Henninghausen et al., 1991; Hobbs et al., 1992; Rijnkels et al., 1995). The αs1-, the β-, the αs2-, and the κ-casein genes are arranged in a clustered region of around 200 kb in mouse chromosome 5, rabbit chromosome 12, and chromosome 4 of ruminants (Geißler et al., 1988; Gellin et al., 1985; Hayes et al., 1992; Threadgill and Womack, 1990). Similarities found in the primary nucleotide sequences and in the genomic organization of the first exons of the αs1-, the β- and the αs2-casein genes suggest a common evolutionary origin for these three genes (Mercier and Villette, 1993; Yu-Lee et al., 1986). The κ-casein gene, in turn, seems to be more diverse, sharing some characteristics with the fibrinogen gene (Jollès et al., 1974).

It is conceivable that a locus control region commands the regulated expression of all casein genes, as found for the cluster of globin genes, where a distant 5′ element controls the regulated expression of each member of the globin cluster (Grosveld et al., 1987; Strouboulis et al., 1992). However, regulated expression of the β-casein gene, which is located between the αs1- and the αs2-casein genes, seems to be independent of the presence of its neighbour genes (Rijnkels et al., 1995). Stable transfection experiments performed in a mouse mammary cell line demonstrated that proximal sequences of the bovine β-casein gene promoter contain the necessary elements to account for transcriptional events regulated by extracellular matrix components and a defined balance of lactogenic hormones.

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(Schmidhauser et al., 1990, 1992). Deletional analysis showed that the levels of expression of the reporter genes used in these experiments were highly dependent on the presence of the proximal 1.7 kb of the bovine β-casein gene promoter (Ball et al., 1988; Devinyo et al., 1988; Doppler et al., 1989, 1990; Eisenstein and Rosen, 1988; Schmidhauser et al., 1990, 1992) in agreement with the fact that several cis-acting elements and their corresponding transcriptional enhancers and repressors have been identified within this area (Altioik and Groner, 1993; Meier and Groner, 1994; Schmitt-Ney et al., 1992). Nonetheless, reporter genes driven by a 1.7-kb bovine β-casein promoter were transcriptionally inactive in most transgenic mice (Maga et al., 1994) or rats (Ninomiya et al., 1994) generated.

To analyze the ability of larger 5’ flanking segments of the bovine β-casein gene to recapitulate the cell-specific and temporal-restricted pattern of expression of a reporter gene in the context of a live mammal, we generated transgenic mice carrying a fusion gene containing 3.8 kb of the bovine β-casein gene promoter driving the expression of the human growth hormone (hGH) gene. Here we report that these promoter sequences were capable of directing a high-level expression of hGH exclusively to the epithelial cells of the lactating mammary gland. In addition, we show that the developmental expression pattern through pregnancy and lactation followed that of the bovine β-casein gene rather than that of its murine homologue.

**MATERIALS AND METHODS**

**Transgene Construct**

The unique Bam HI site present in the plasmid Bluescript SK ± (Stratagene, La Jolla, CA) was eliminated by digestion with Bam HI followed by a fill-in with Klenow DNA polymerase and a final ligation of the blunt ends. A 2.2-kb Sal I–Eco RI fragment from plasmid p0GH (Nichols Institute Diagnostics, San Juan Capistrano, CA) containing the entire transcriptional unit of the human growth hormone (hGH) gene was isolated and subcloned into the Bam HI-less generated plasmid giving pBS-hGH. Finally, a Sal I–Bam HI fragment containing 3815 bp of the 5’ flanking region of the bovine β-casein gene plus 40 bp of the first untranslated exon was excised from plasmid pbjcas-3815+40/CAT (Schmidhauser et al., 1990; kindly provided by Connie A. Myers) and subcloned in front of the hGH gene to generate pbjcas/hGH.

**Production of Transgenic Mice**

Transgenic mice were generated by pronuclear microinjection of B6CBF2 zygotes through glass capillary micropipettes attached to a manual micromanipulator (Leica, Germany). Fertilized eggs were isolated at 9 to 10 A.M. as described elsewhere (Low, 1992) and observed under differential interference contrast optics of an inverted microscope (FS Labovert, Leica, Germany) and microinjected into the most visible pronucleus with approximately 500 molecules of the transgene dissolved in 1 µl of a sterile solution containing 5 mM Tris HCl (pH 7.4) and 0.1 mM EDTA (pH 8.0). The transgene was prepared as follows: A 6-kb fragment was released from plasmid p3.8bjcas/hGH by a Sal I and Not I double digestion, separated by agarose electrophoresis, collected by electroelution and purified through an ion-exchange Elutip-D column (Schleicher & Schuell, Keene, NH). After microinjection, eggs were transferred to the oviduct of pseudopregnant Swiss Webster females 6 to 10 weeks old.

**Transgenic Mouse Identification**

Screening for positive transgenic mice was performed by means of the polymerase chain reaction (PCR) on genomic DNA extracted from tail biopsies. Primers 149 (5’-CCTGTTATTCTGGTG TG-3’) and 150 (5’-AGTTGTTGCGGGAGTGG-3’) amplify a transgene specific band of 412 bp (see Fig. 1A). DNA was amplified into a 1605 Air Thermo Cycler (Idaho Technology, Idaho Falls, ID) as follows: a first denaturation step at 94°C during 5 min, followed by 35 cycles at 94°C for 0 sec, 60°C for 10 sec, and 72°C for 15 sec, with a final elongation step at 72°C for 10 min. Positive founder mice were confirmed by Southern blot analysis of Eco RI-digested genomic DNA extracted from tail biopsies. DNA fragments were separated by submarine electrophoresis in a 0.7% agarose gel and then transferred to a Zeta-Probe nylon membrane (Bio-Rad Hercules, CA). Blot hybridization was carried out using a 0.84-kb Eco RI–Hind III fragment isolated from the bovine β-casein promoter (see Fig. 1) and radiolabeled by random priming (GIBCO-BRL, Bethesda, MD) with [a-32P]dCTP (DuPont-NEN, Boston, MA). Hybridization was performed at 65°C during 16 hr in a solution containing 6× SSC, 25 mM phosphate buffer (pH 7.2), 5× Denhardt’s, 0.5% SDS, 1 mM EDTA (pH 8.0), and 100 μg/ml denatured salmon sperm DNA. Blots were then washed in 2× SSC and 0.1% SDS at room temperature for 15 min twice and then in 0.1× SSC and 0.1% SDS at 60°C for 30 min and finally exposed to x-ray film (Kodak, Rochester, NY) using intensifying screens at −70°C. The transgene copy number of each line was estimated by a scanning densitometric analysis (Gel-Works 1D; UVP, England). Values were interpolated into a concentration curve prepared with mouse control DNA carrying known amounts of the transgene.

**hGH Determination in Milk and Serum by Radioimmunoassay**

At day 11 of lactation, the mothers were separated from their pups, anaesthetized with 300 mg/kg IP of 2,2,2-tribromoethanol, and then injected IP with 0.6 IU of oxytocin (Syntocinon, Sandoz, Argentina). Milk samples were collected in capillary tubes after a gentle massage of the glands 5 min after receiving oxytocin and then stored at −70°C until used. Serum samples were obtained by centrifugation (14,000 rpm × 10 min) of blood obtained by cardiac puncture in mice anaesthetized as above. Sera was stored at −70°C until used. Concentrations of hGH in milk and serum were deter-
mined by radioimmunoassay (RIA) using a specific hGH kit (Diagnostic Product Corporation, Los Angeles, CA) according to the manufacturer’s instructions. This RIA quantifies hGH at concentrations ranging from 1.4 to 30 ng/ml. Milk samples were diluted 1:5 in a solution containing 125 mM NaCl, 25 mM Tris HCl (pH 7.4), 5 mM KCl and 2 mM phenylmethylsulfonylfluoride (PMSF). Samples were centrifuged at 2000 rpm for 5 min and the fat layer carefully removed. The resulting skim milk was clarified once again by centrifugation.

**PAGE/SDS and Western Blot Analysis**

Milk was diluted 1:10 in a buffer containing 10 mM Tris HCl (pH 8.0) and 10 mM CaCl₂. Lactoserum fractions were obtained from the diluted milk by centrifugation at 14,000 g for 45 min. Total milk proteins and proteins from the lactoserum fraction were separated on a 15% polyacrylamide gel as described elsewhere (Laemmli, 1970) using a Mini-Protean II dual slab cell (Bio-Rad, Hercules, CA). Protein bands were visualized by staining gels with Coomassie Brilliant Blue. For immunoblots, total milk proteins were separated under the same conditions as described above and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Membranes were blocked overnight in TBS (25 mM Tris HCl, pH 8.0, 175 mM NaCl) containing 5% defatted powder milk. As a first antibody, a rabbit anti-hGH serum was used (kindly provided by Dr. Carlos Melo) in a 1:1000 dilution added to the blocking solution. Incubation proceeded for 2 hr at room temperature. The blot was then washed three times with TBS/0.05% Tween 20 at room temperature for 5 min. A peroxidase-conjugated goat antirabbit IgG (Amersham, UK) diluted 1:2500 in blocking solution was used as the second antibody during 1 hr at room temperature. The blot was washed twice with TBS/ 0.05% Tween 20 at room temperature for 5 min and then once with TBS for 5 min. Chemiluminescence detection was performed with Renaissance reagents (DuPont-NEN, Boston, MA) according to the manufacturer’s instructions.

**Northern Blot Analysis**

Total RNA was extracted from the following organs of lactating transgenic mice: hypothalamus, brain cortex, cerebellum, rest of brain, mammary gland, pituitary gland, heart, spleen, skin, skeletal muscle, and liver using the guanidine thiocyanate–acid phenol method (Chomczynski and Sacchi, 1987). To follow the expression of the transgene during mammary gland development, total RNA was isolated from this tissue at different stages of gestation (postcoitum days 7, 10, 13, and 17) and lactation (postnatal days 1, 11, and 21). Then 10–20 µg of total RNA dissolved in 80% formamide was incubated at 65°C for 15 min and then loaded into a 1.2% agarose/4% formaldehyde gel in a buffer containing 4% formaldehyde, 20 mM MOPS (pH 7.0), 8 mM sodium acetate, and 1 mM EDTA (pH 8.0) and subjected to submarine electrophoresis at 3.8 V/cm. Gels were capillary transferred to Magnacharge nylon membranes (Micron Separations, Inc., Westborough, MA), UV cross-linked (UV Stratalinker 1800; Stratagene, La Jolla, CA), and then sequentially hybridized with probes corresponding to exon 5 of the hGH gene, exon 7 of the mouse β-casein gene, and a complete cDNA of mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH). All probes were radiolabeled with [α-32P]dCTP by random priming (GIBCO-BRL, Bethesda, MD). Hybridizations were performed following the same conditions as described above for the Southern blots. Previous to the use of a different probe, blots were stripped in a boiling solution containing 0.1× SSC and 0.1% SDS for 10 to 30 min.

**In Situ Hybridization Studies**

Mammary glands were dissected at day 11 of lactation and fixed in 4% (w/v) paraformaldehyde overnight at 4°C. Tissues were dehydrated through a series of ethanol solutions (70%, 95% and 100%), infiltrated with xylene, and embedded in paraffin. Serial 6-µm sections...
were cut from paraffin blocks, mounted on polylysine-coated slides, and stored at room temperature until used. Sections were deparaffinized, rehydrated in a graded series of ethanol solutions (100%, 95% and 70%), fixed in neutral phosphate-buffered/10% formalin (Sigma, St. Louis, MO) at room temperature for 30 min, and washed three times with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM CaCl₂, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄) for 10 min at room temperature followed by three washes with 2× SSC (1× SSC: 0.15 M NaCl, 15 mM Na citrate, pH 7.2) for 10 min at room temperature. Slides were drained 5–10 min and then incubated under Parafilm coverslips with 35–50 µl of a solution containing 50% formamide, 4× SSC, 1× Denhardt’s, 250 µg/ml yeast tRNA, 500 µg/ml sheared and denatured salmon sperm DNA, 10 mM dithiothreitol, and 10% dextran sulfate at 37°C for 1 h. Slides were drained again and incubated overnight at 37°C with prehybridization solution containing 11,000–20,000 cpm/µl of radiolabeled oligonucleotide probe. Slides were washed four times with 1× SSC and 1 mM dithiothreitol for 15 min at room temperature followed by four washes with 2× SSC and 50% formamide for 15 min at 37°C. Finally, slides were washed three times with 1× SSC for 30 min at room temperature. After a quick rinse in water, slides were dehydrated through 5 min steps in ethanol 70%, 95%, and 100%, dried at room temperature and exposed to an autoradiography film for 7 to 10 days. After developing the film, slides were dipped in Kodak NTB2 emulsion (diluted 1:1 in distilled water) and exposed for 30 days. Slides were developed, counterstained with neutral red (Sigma), and coverslipped underwater (Permount, Fisher, Pittsburgh, PA). Radiolabeled probes were generated by incubating 5 pm of synthetic oligodeoxynucleotides (30-mers) with 50 pm of [α-35S]dATP (1300 Ci/mmol; DuPont-NEN, Boston, MA) and 50 units of terminal deoxynucleotidyltransferase (GIBCO-BRL, Bethesda, MD). To detect the transgenic hGH mRNA, we used a 30-mer antisense to hGH exon 4 (5'-TGGTTGCGGAGACACTCTTGAG-GAACTGCA-3'), whereas to detect the endogenous mouse β-casein mRNA, we used a 30-mer antisense to mouse β-casein exon 7 (5'-CAGGAAATCGAGACCCAG-CCAGAGATGCGA-3'). Slides were analyzed in a microscope coupled to a TV camera. mRNA amounts were quantified by counting silver grains on random areas of mammary gland sections as observed on a TV screen. One hundred independent determinations were taken from each mammary gland.

RESULTS

Generation of Transgenic Mice

To study the ability of 3.8 kb of the bovine β-casein gene promoter to drive the expression of a reporter gene to the lactating mammary gland, we constructed 3.8βcas/hGH. This chimeric gene contains 3815 bp of the 5' flanking region of the bovine β-casein gene together with 40 bp of its first exon followed by the hGH structural gene (Fig. 1A). Microinjection of this construct into fertilized mouse zygotes gave three founder transgenic mice, two females (3 and 50) and one male (45) initially identified by amplification of a transgene specific sequence (see Fig. 1A) and further confirmed by Southern blot hybridization (see Fig. 1B, C). In Eco RI-digested genomic DNA obtained from tail biopsies, hybridization with an internal probe of the bovine β-casein promoter revealed the presence of an expected 2.5-kb band that allowed an estimation of the number of copies of the transgene for each of the three lines (see Fig. 1B). Scanning densitometry established that lines 3 and 45 have approximately 2 to 3 copies, whereas line 50 has between 6 to 10 copies. When using a Pvu II hGH internal fragment as a hybridization probe, the expected 2.3 kb band was detected. In addition, an extra 1.6-kb band and a high-molecular-weight band were evident on genomic DNA from line 3 suggesting an intrarecombination event (see Fig. 1C). Breeding lines were transmitted as a single locus in a mendelian fashion.

hGH in the Milk of Transgenic Mice

The advantage of using a secreted protein as a reporter gene product targeted to the epithelial cells of the mammary gland is that its presence may be monitored in the intact live animal through a noninvasive approach. Milk samples were obtained from founder females 3 and 50 and from F1 females of line 45 on day 11 of lactation, and the concentration of hGH was determined by radioimmunoassay using a specific antibody directed against hGH. Initial determinations showed 0.42 ± 0.07 mg/ml of hGH in milk from founder 3 and 1.80 mg/ml in milk from founder 50. In contrast, hGH levels in F1 mice from line 45 only reached 100 to 125 ng/ml (Table 1). All these values proved to be maintained along three successive generations and were doubled when analyzed in milk from homozygous transgenic females (see Table 1).

| Table 1. Radioimmunoassay of hGH in Milk of Transgenic Mice |
|-----------------|------------------|
| Line | Transgene copy number | hGH concentration in milk (mg/ml) |
| 3 (+/−) | 2–3 | 0.382 ± 0.027 (10) |
| 3 (+/+) | 2–3 (×2) | 0.764 ± 0.228 (4) |
| 50 (+/−) | 6–10 | 2.22 (2)* |
| 45 (+/−) | 2–3 | 1.13 × 10−4 (2)† |
| nt | 0 | UD (2) |

Note: Values express the mean ± SEM when more than three samples were analyzed. The number of milk samples is indicated in parentheses. Each milk sample was taken from a different mouse. (+/−), transgenic mice heterozygous for the transgene; (+/+), transgenic mice homozygous for the transgene; nt, nontransgenic; UD, undetectable (<1.4 × 10−6 mg/ml).

*Individual values were 1.80 and 2.63 mg/ml.
†Individual values were 1.04 × 10−4 and 1.21 × 10−4 mg/ml.
SDS of denatured protein samples taken from total milk or from the lactoserum fraction. A 22-kDa band corresponding to hGH was detected by Coomassie Blue staining in 0.3 µl of milk and its corresponding lactoserum fraction obtained from line 50 (Fig. 2A). Due to the lower hGH concentration in the milk of line 3, it was necessary to load the protein content present in 2 µl of milk to detect the transgenic protein in the lactoserum fraction (see Fig. 2B). These bands comigrated with mouse growth hormone (mGH) observed in the pituitary protein extract of a nontransgenic mouse. The fact that hGH is still present in the lactoserum fraction suggests that the transgenic protein does not get included into micelles but remains soluble in the milk. A Western blot analysis using an hGH antiserum confirmed these data and revealed the presence of an additional band of 44 kDa in milk from line 3 (see Fig. 2C).

Spatial Pattern of Transgene Expression

To study the tissue-specific expression of the transgene, we analyzed hGH mRNA contents in the lactating mammary gland of transgenic mice from lines 3 and 50, which proved to secrete high levels of hGH into the milk. Total RNA was extracted at midlactation (day 11) and subjected to a Northern blot hybridization using a probe corresponding to almost the entire hGH exon 5. As shown in Fig. 3, high levels of a 1-kb band corresponding to hGH mRNA were found only in the lactating mammary gland of both transgenic lines, whereas mouse β-casein mRNA was found in mammary glands taken from transgenic and nontransgenic lactating females. Line 3 showed, in addition, a larger band of 1.44 kb that probably corresponds to a transcriptional product from one of the extra transgenic bands detected in this line after the analysis of Southern (see Fig. 3). This larger hGH-related mRNA found in line 3 probably accounts for the higher-molecular-weight hGH-like immunoreactive protein observed in the Western blot study.

To determine whether the 3.8-kb bovine β-casein promoter targets transgenic hGH expression exclusively to the mammary gland, we isolated total RNA samples from various tissues of transgenic females on lactation day 11 and performed a Northern blot hybridization analysis. Similarly to what we found for the mouse β-casein gene, no tissue other than the mammary gland showed expression of the hGH gene (Fig. 4A, B), even after long time exposures (data not shown), demonstrating a restricted cell-specific expression pattern. Only the pituitary gland from both transgenic and nontransgenic mice showed the presence of a band that corresponds to the mouse growth hormone mRNA that is slightly shorter than its human homologue (see Fig. 4A, B).

In order to analyze the possibility of a minor ectopic expression in tissues capable of secreting proteins into the blood circulation, we determined the presence of hGH in serum samples by radioimmunoassay (Table 2). Serum samples taken from young adult males and from

![Fig. 2. Detection of hGH in the milk of transgenic mice from lines 3 and 50. (A) 0.3 µl of milk or its corresponding lactoserum fraction was resolved in a 1.5-mm-thick 15% PAGE/SDS under reducing conditions. 50 µg of a pituitary protein extract from a nontransgenic mouse was included. Gels were stained with Coomassie Brilliant Blue. (B) Idem but for 2 µl of milk and for 150 µg of pituitary protein extract. (C) 0.3 µl of milk was separated by PAGE/SDS, as described above, and then the gel was transferred to a nitrocellulose filter and incubated with an hGH antiserum (nt, nontransgenic).](image-url)
showed very stable serum hGH levels of around 10 ng/ml regardless of their sex or physiologic stage (see Table 2). However, these low levels of hGH did not produce any of the common effects reported in transgenic mice carrying large amounts of hGH in plasma (Brem et al., 1989; Cecim et al., 1995), such as increased body size and weight or reduced fertility. Mice from line 3 were normally fertile and displayed a normal growth curve throughout development and adulthood (data not shown).

**Temporal Pattern of Transgene Expression**

Expression of the casein genes in the mammary gland follows a strict developmental pattern strongly dependent on the hormonal influences that occur during pregnancy and lactation. To study whether the 3.8-kb bovine β-casein gene promoter accounts for this specificity, we analyzed hGH expression in mammary gland tissue obtained from timed pregnant transgenic females. Total RNA isolated from mammary gland biopsies taken at different stages of pregnancy and lactation were subjected to Northern blot analysis. Strong induction of hGH expression occurred on parturition (Fig. 5A). hGH transcript levels were at the threshold of detection at day 17 of pregnancy (see Fig. 5B). From day 17 of pregnancy to day 1 of lactation, hGH expression increased dramatically up to 50-fold (see Fig. 5A, B). In contrast, induction of the endogenous murine β-casein gene occurred in midpregnancy and was followed by a steady increase in expression during the rest of the gestation period and up to day 11 of the lactation period (see Fig. 5A). This developmental pattern of mouse β-casein expression has been described previously, and it is different from that reported in the cow, where β-casein mRNA levels are low during gestation and abruptly increase on lactogenesis (Goodman and Schanbacher, 1991). Therefore, our data indicate that expression driven by a bovine β-casein gene promoter in transgenic mice follows the bovine developmental pattern rather than that found for the mouse gene.

**Cell-Specific Pattern of Transgene Expression**

It has been reported extensively through histochemical studies that tissue-specific promoters capable of targeting the expression of reporter genes to a defined cell type often show a heterogeneous pattern of expression or cellular mosaicism (Faerman et al., 1995; McGowan et al., 1989). To determine the degree of homoge-
neity of the 3.8-kb bovine β-casein gene promoter driving hGH expression to the mammary gland, we performed an in situ hybridization study on paraffin sections obtained from mammary glands of control and transgenic females at day 11 of lactation. Film autoradiography showed that mouse β-casein levels were similar among all mice studied, including a nontransgenic control. The intensity of hGH mRNA signal varied between lines and genotypes, although showing, in every case, a uniform pattern of expression (Fig. 6). Sections were then dipped into a sensitive emulsion, and the silver grains were counted under an upright microscope. We observed that most of the cells expressed the transgene regardless of the total level of transgenic expression throughout all the surface of the tissue. This result suggests that the 3.8-kb bovine β-casein promoter drives a homogeneous expression to the mammary gland. The overall difference of expression between lines or genotypes is due to a variable expression within each cell rather than a different penetrance of the transgene in the tissue. Finally, we found a very high correlation between the density of silver grains present on transgenic mammary gland sections and the concentration of hGH present in the milk of the same mouse determined by RIA (Fig. 7).

DISCUSSION

Stable transfection experiments have demonstrated that proximal sequences of the bovine β-casein gene promoter were able to drive the expression of a reporter gene selectively to a mammary-derived cell line and that these transcriptional events were highly dependent on extracellular matrix components and a defined balance of lactogenic hormones (Schmidhauser et al., 1990). However, cell culture systems proved to be in several cases too permissive to study the regulated expression of mammalian genes. Promoters that seemed to drive a regulated expression of reporter genes to a specific differentiated cell line often failed to initiate transcription in the equivalent cell type of transgenic mice or were shown to be transcriptionally active in ectopic sites (Günzburg et al., 1991; Low et al., 1986; Zack et al., 1991). In fact, production of transgenic animals carrying 1.79 kb (Maga et al., 1994) or 1.72 kb (Ninomiya et al., 1994) of the bovine β-casein gene

### TABLE 2. RIA Determination of hGH in Serum of Transgenic Mice

<table>
<thead>
<tr>
<th>Line</th>
<th>Virgin</th>
<th>Pregnant</th>
<th>Lactating</th>
<th>Males</th>
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<tr>
<td>Females</td>
<td></td>
<td></td>
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<tr>
<td>3 (t)</td>
<td>10.33 ± 0.92 (13)</td>
<td>7.49 ± 0.99 (4)</td>
<td>10.18 ± 0.54 (9)</td>
<td>9.92 ± 0.99 (5)</td>
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<tr>
<td>50 (t)</td>
<td>UD (6)*</td>
<td>4.49 (2)*</td>
<td>UD (1)</td>
<td>UD (7)</td>
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<tr>
<td>nt</td>
<td>UD (7)</td>
<td>ND</td>
<td>UD (4)</td>
<td>UD (3)</td>
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Note: Values express the mean ± SEM when more than three samples were analyzed. The number of serum samples analyzed is indicated in parentheses. t, transgenic; nt, nontransgenic; UD, undetectable (<1.4 ng/ml); ND, not determined.

*One of the serum samples, not included in this table, measured 8.51 ng/ml.
†Individual values of both serum samples were 3.89 and 5.08 ng/ml.

![Fig. 5.](image-url) Temporal expression of the 3.8kbβcas/hGH transgene in the mammary gland of transgenic mice. Northern blot analysis of total RNA isolated from mammary gland tissue of transgenic mice from line 3 (A), from line 50 (B), and from nontransgenic (nt) mice (A). Mammary gland biopsies were obtained at different stages throughout pregnancy and lactation: virgin (V), pregnant (P) at 7, 10, 13, and 17 days postcoitum, and lactating (L) at 1, 11, and 21 days postpartum. 10 µg of total RNA was resolved by denaturing gel electrophoresis, transferred to Nylon filters, and sequentially probed with an exon 5 fragment of the hGH gene, an exon 7 fragment of the mouse β-casein gene, and a cDNA of the mouse G3PDH.
promoter failed to show transgenic expression in the mammary gland in most of the cases despite the fact that these promoters had proven previously to be selectively active in a mouse mammary-derived cell line (Schmidhauser et al., 1990). Here we report that 3.8 kb of the bovine $\beta$-casein gene promoter have the ability to induce an accurate spatial and temporal pattern of expression in the mammary gland of lactating transgenic mice. From all tissues analyzed, only the lactating mammary gland displayed transcriptional activity of the reporter gene that was further evidenced by the presence of relatively high levels of the transgenic protein in the milk of two of the three pedigrees generated. To date, the only other example of an accurate expression driven by bovine $\beta$-casein sequences was obtained in transgenic mice carrying an entire 33-kb genomic fragment of this gene including 16 kb of 5' flanking sequences together with 8 kb of 3' flanking sequences. This construct allowed high expression levels of bovine $\beta$-casein in almost 50% of the transgenic mouse lines analyzed (Rijnkels et al., 1995). We also detected low levels of hGH in the serum of mice from line 3 regardless of their sex, age, or lactating status. Because the $\beta$-casein gene is expressed in cytotoxic T-lymphocytes (Grusby et al., 1990), it is conceivable to find recombinant hGH in the serum as a secreted product from those cells, although a minor ectopic expression from other tissues cannot be ruled out. Nevertheless, the hGH present in the serum did not seem to interfere with the normal physiology of these animals, since no changes in their growth rate or fertility were observed.

$\beta$-Casein gene expression follows a strict developmental pattern that progresses together with the growth and maturation of the lactating mammary gland. Whereas in rodents $\beta$-casein initiates its expression at midgestation (Henninghausen et al., 1991), in cattle $\beta$-casein mRNA only peaks on lactation (Goodman and Schanbacher, 1991). In contrast to what we expected, expression from a bovine $\beta$-casein promoter in transgenic mice followed the bovine temporal program rather than the murine. Transgenic expression was evident at gestation day 17.5 but showed elevated levels only on parturation. This interspecies difference could be explained by an early acting transcription factor that would bind to its specific cis-element only present in the mouse promoter. An alternative explanation for this temporal discrepancy could involve the participation of a transcriptional repressor of the $\beta$-casein gene that slowly becomes inactive on midgestation. A higher affinity of this repressor for the bovine cis-acting element would result in a more prolonged transcriptional inhibition of the bovine promoter. For example, the $\beta$-casein gene repressor YY1 that belongs to the family of GL1-Krüppel zinc finger transacting factor was proposed to act as an activator or as a repressor depending on the local concentration of the factor or the nature of the cis-binding site (Meier and Groner, 1994).

**Fig. 6.** In situ hybridization of mouse $\beta$-casein and 3.8$b$;cas/hGH transgene in mammary gland from lactating females. Paraffin sections of mammary glands taken from mice at midlactation (11 days postpartum) were probed with an $[^{35}S]a$-dATP tail labeled oligodeoxynucleotide antisense to the exon 7 of the mouse $\beta$-casein gene (m$\beta$-casein) or antisense to the hGH exon 4 of the hGH gene. Slides were exposed on x-ray films for 7 to 10 days. nt (-/-), non transgenic mouse; 3 (+/-), mouse from line 3 heterozygous for the transgene; 3 (+/+), mouse from line 3 homozygous for the transgene, 50 (+/-), mouse from line 50 heterozygous for the transgene.

**Fig. 7.** Correlation curve between transgenic hGH mRNA levels in epithelial cells of the mammary gland and transgenic hGH levels found in the milk. hGH mRNA amounts were estimated by in situ hybridization as silver grains per square millimeter. Each mRNA quantitation corresponds to the mean of one hundred independent measures taken at random from four different sections for each gland. hGH levels were determined by RIA as shown in Table 1. ●, mouse from line 3 heterozygous for the transgene; ▲, mouse from line 3 homozygous for the transgene, ■, mouse from line 50 heterozygous for the transgene.
Another interesting feature observed when analyzing the transcriptional activity of the 3.8-kb bovine β-casein promoter is the homogeneous pattern of expression of the reporter gene throughout all mammary gland lobules. In contrast to many other promoters that show a cellular mosaicism for the expression of transgenes, including that from the ovine β-lactoglobulin gene (Faerman et al., 1995), the 3.8-kb bovine β-casein promoter induced expression of the hGH gene in all epithelial cells regardless of the total transgenic expression level or whether the tissue corresponded to mammary glands from heterozygous or homozygous transgenic mice. Cellular mosaicism, which is often evidenced by an incomplete or patchy pattern of gene expression in contrast to the homogeneous pattern observed for endogenous genes, is probably due to a mosaic pattern of transgene methylation and is more commonly observed for incomplete promoters that also manifest a low expression penetrance (Bradl et al., 1991; Faerman et al., 1995; Liu et al., 1992; McGowan et al., 1989).

The reproducible accurate pattern of developmental and tissue-specific expression of the 3.8bpicas/hGH transgene is observed together with variable quantitative levels of transgenic expression. This suggests that the transcriptional strength of the 3.8 kb bovine β-casein promoter is dependent on the site of integration of the transgene within the mouse genome. cis-acting elements present in the proximities of the transcriptional unit of a gene are necessary to control four major aspects of its appropriate expression, and these are (1) the spatial pattern, (2) the temporal or developmental pattern, (3) the hormonal regulation pattern, and (4) the quantitative levels of expression in each cell and moment. The results presented here show that the 3.8-kb bovine β-casein promoter provides a highly efficient control to the first three aspects of gene regulation but fails to produce appropriate or constant levels of transgenic expression among the various pedigrees. This characteristic suggests that the frequency of occurring transcriptional events is strongly dependent on the chromosomal context around the transgene integration site. Specific trans-acting factors induced in the epithelial cells of the lactating mammary gland are able to recognize their corresponding cis-acting sites but only when they become readily available by an open chromatin configuration (Al-Shawil et al., 1990). Position independence and copy number dependence are two parameters infrequently achieved when analyzing expression levels of transgenes. To this end, transgenes need to harbor DNA elements that could insulate their transcriptional units from influencing regions present in the vicinities of the integration site. In a few cases such as the lysozyme gene, matrix attachment regions (MARs) have been identified and proved to give position independence of transgenes by becoming the active borders between transcriptionally active and inactive chromatin (Bonifer et al., 1990; Phi-Van and Strätling, 1988). However, attempts to use these lysozyme gene MARs to insulate heterologous genes proved to be only partially effective (Barash et al., 1996; McKnight et al., 1996).

To overcome the transcriptional position effects of transgenes driven by bovine β-casein promoter sequences, it will be necessary to identify locus control regions (LCR) that probably command the transcriptional activity of every member of the casein gene cluster, as was found for the family of globin genes (Grosfeld et al., 1987; Strouboulis et al., 1992). Availability of a putative LCR acting in concert with the elements present in the 3.8-kb bovine β-casein promoter could provide a very useful tool to recapitulate the entire qualitative and quantitative aspects of the regulated expression of this gene.

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REFERENCES


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