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The bovine α-lactalbumin promoter directs expression of ovine trophoblast interferon in the mammary gland of transgenic mice

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A hybrid construct derived from ovine trophoblastin cDNA and bovine α-lactalbumin-encoding gene, was injected into the pronuclei of mouse eggs. In one of the resulting transgenic mouse lines, expression of the hybrid construct was detected and found to be limited to the mammary gland of lactating females which secreted active ovine trophoblastin. This strongly suggests that important *cis*-acting DNA sequences involved in tissue-specific expression of the bovine gene are located within the second half of the 3′ untranslated region, or/and the proximal 5′ and 3′ regions flanking the transcriptional unit.

α-Lactalbumin; Trophosblast interferon; Bovine; Ovine; Transgenic mouse; Milk composition

1. INTRODUCTION

The entire bovine α-lactalbumin (bαLa) gene and the flanking DNA sequences have been isolated and characterized in our laboratory [1]. Expression of this gene in several lines of transgenic mice was stage- and tissue-specific, and in two of them, the yield of bαLa in milk was close to that of its endogenous counterpart [2], indicating the occurrence of *cis*-acting mammary regulatory elements within the transgene. Promoter sequences of various milk protein genes including β-lactoglobulin, whey acidic protein (WAP), β- and αs1-casein have been used to direct expression of foreign proteins in the mammary gland of transgenic mice (see [3] for review).

In order to demonstrate the potential usefulness of the bαLa promoter for driving the expression of a linked gene in mamamary gland, the ovine trophoblastin (oTP) cDNA was chosen as reporter, since this αII-like interferon (IFN-αII) can be detected and quantified easily. Trophoblast proteins play a major role in the establishment of pregnancy in ruminants by blocking the pulsatile secretion of prostaglandin $F_{2\alpha}$ by the uterus and thereby ensuring the maintenance of the corpus luteum function (see [4] for review).

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Abbreviations: bαLa, bovine α-lactalbumin; oTP, ovine trophoblastin; IFN, interferon

2. MATERIALS AND METHODS

2.1. *Materials*

All enzymes, RNase protection kit and oligo-labelling kit were purchased from Boehringer Mannheim. [α-^{32}P]dCTP was obtained from Amersham. Other chemicals came either from Sigma or Poly-labo.

2.2. *Establishment of the hybrid construct*

A 3.2 kb *Hin*dIII/*Pst*I genomic fragment comprising the bαLa transcription unit with 740 bp and 450 bp of 5′- and 3′-flanking region [1], respectively, was cloned into the corresponding sites of the pPolyIII-I poly-linker [5]. Most of the transcription unit of the bovine gene was excised, leaving only 153 bp of the 3′ untranslated region, and replaced by the oTP cDNA, shortened of 60 bp at the 3′ end [6], as illustrated in Fig. 1. The 2.3 kb insert was released by digestion with *Xho*I and purified as already described [2].

2.3. *Micro-injection, transfer of mouse eggs and identification of transgenic mice*

Microinjection was performed on pronuclei of (C57BL/6 × CBA) F2 hybrid eggs [7]: 1041 eggs were each injected with approximatively 200 copies of the linearized hybrid gene and re-implanted into pseudopregnant recipient mice; 66 mice were born, of which 20 died accidentally soon after birth (cannibalism, death of foster mothers). Transgenic mice were identified by Southern analysis of their genomic DNA as described in [2] and illustrated in Fig. 2.

2.4. *RNA isolation and analysis*

Total RNA was isolated after homogenization of tissues in a guanidium thiocyanate solution as described in [8]. RNase protection was performed according to Sambrook et al. [9] and as illustrated in Fig. 3.

2.5. *Milk protein analysis*

Milk was collected from nursing mice, 12 days after parturition as previously described [10]. Milk samples were defatted by centrifugation after a 1/5 dilution in distilled water [2], and tested in an antiviral interferon bioassay based on protection against cytopathic effects of

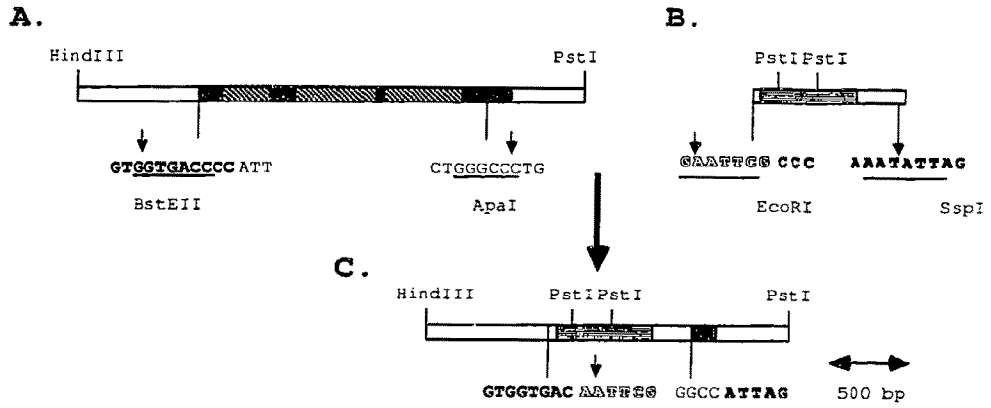

Fig. 1. Construction of the hybrid gene. A. Structure of the bαLa gene: white, black, hatched and grey segments represent 5′ or 3′ flanking regions, the coding frame, introns and 5′ or 3′ untranslated regions, respectively. Positions and sequences of the two restriction sites, used to remove part of the transcription unit, are indicated below (bold-faced nucleotides refer to the 5′-flanking region, recognition sites are underlined and cutting sites are indicated by arrows). B. Structure of the oTP cDNA: light-grey and striped segments represent the 5′ or 3′ untranslated regions and the coding frame, respectively. Positions and sequences of the two restriction sites used for inserting the cDNA between the two bαLa-derived fragments are indicated below (the *Eco*RI site is part of the linker used for cDNA cloning [6], recognition sites are underlined and cutting sites indicated by arrows). C. Structure of the hybrid gene. All fragments were end-blunted before ligation. Sequences of ligation sites are indicated below. The arrow indicates the new potential CAP site.

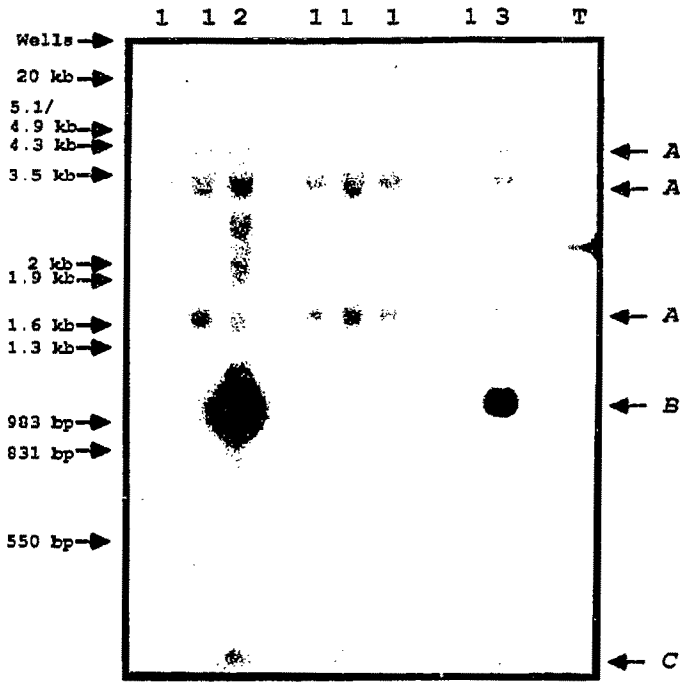

Fig. 2. Characterization of transgenic mice by Southern blot analysis. Southern blot experiments were carried out on *Pst*I-digested genomic DNA as described in [2]. Right margin: (A) restriction fragments derived from endogenous IFN-related sequences; (B) 1.1 kb *Pst*I restriction fragments corresponding to the 3′ end of the transgene and to its 5′ end in the case of multiple copies arranged in a head-to-tail fashion (see Fig. 1); (C) 200 bp internal *Pst*I fragment from the transgene. Left margin: λ-*Hin*dIII/*Eco*RI size markers. Lane T: lincarized transgene in amount equivalent to 1 copy per cell, lane 1: DNA from non-transgenic mice, lane 2: DNA from transgenic offspring of founder mouse 33, lane 3: DNA from transgenic offspring of founder mouse 13. In lanes 2 and 3, hybridizing fragments, other than those mentioned above, might correspond to junction fragments.

vesicular stomatitis virus (VSV) on Madin Darby Bovine Kidney (MDBK) cells [11]. IFN titers were calculated relative to that of a porcine IFN-α1 standardized with human reference IFN NIH, GA, 23 902 530 (NIH Bethesda MD) [12].

3. RESULTS

3.1. *Generation of transgenic mice*

Eight transgenic mice, including three females, 13, 24 and 33, were detected out of the 46 screened by Southern analysis of their tail-purified genomic DNA (Fig. 2 and Table I). Multiple copies, ranging from 2 to around 20, were arranged in a head-to-tail fashion. Unfortunately, two founder mice, 21 and 24, died before giving birth to any transgenic offspring, one, 43, was found to be sterile and only four out of five left transmitted the transgene to their offspring. None of those had multiple integration sites as judged from Southern blotting DNA analysis of their progeny.

3.2. *Expression of the transgene*

Expression of the transgene was only found in the mammary gland of lactating transgenic offspring of founder mouse 13: specific RNAs were detected in mammary tissue by RNase protection (Fig. 3) and an interferonic activity of about 200000 U/ml was found in milk samples (Table I), which corresponds to an oTP concentration close to 1 μg/ml, assuming an IFN activity of $2–3 \cdot 10^8$ U/mg of oTP [13].

4. DISCUSSION

Mammary tissue-specific expression of both the entire bαLa gene [2] and the derived hybrid gene indicates that some, if not all relevant *cis*-acting DNA elements

Table I

Summary of transgene expression in the different lines

Line	Copies integrated	Transmission founder mice	Integration sites	Multiple copies arrangement	Expression in transgenic offsprings	
					mRNA/Tissue specificity	Protein in the milk (U/ml)
13	05	2/14 (14.3%)	1	head-to-tail	yes/yes	~200000
19	15	7/16 (43.7%)	1	head-to-tail	no	0
21	02	0/6	n.d.	head-to-tail	n.d.	n.d.
24	10	n.d.	n.d.	head-to-tail	n.d.	n.d.
26	15	0/36 /0%)	n.d.	head-to-tail	n.d.	n.d.
33	20	5/12 (41.6%)	1	head-to-tail	no	0
36	20	2/2 (100%)	1	head-to-tail	no	0
43	05	Sterile	n.d.	head-to-tail	n.d.	n.d.
Mouse						0

Numbers of integrated copies were estimated with internal standards as described in [2]. n.d., not determined; no, undetectable levels. Protein concentrations were estimated as described in Section 2. ~: about.

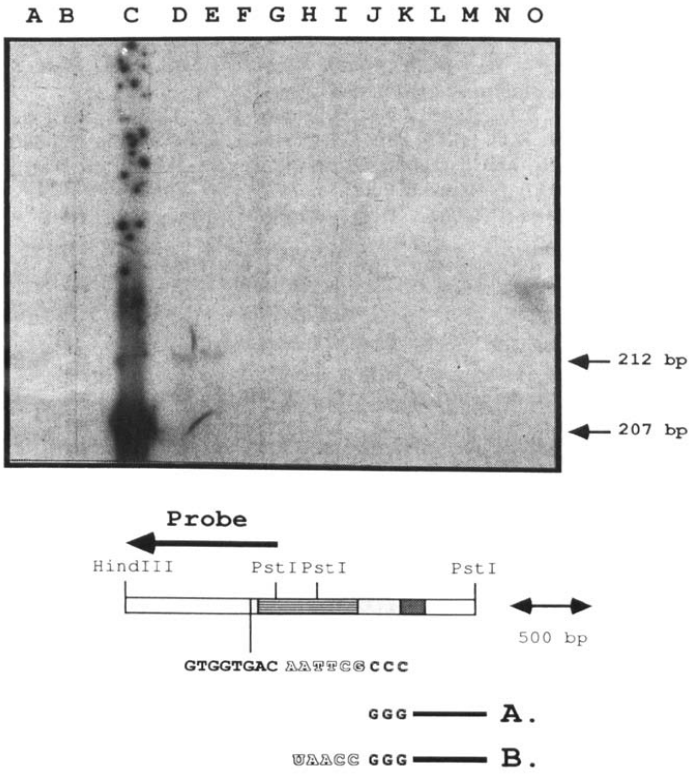

Fig. 3. Transcript analysis by RNase protection. Each sample contained 20 μg of RNA extracted from: lactating mammary gland (lane A) and liver (B) from a non-transgenic mouse; day 16 sheep embryo (C); lactating mammary glands (D,E), liver (F), spleen (G) and salivary gland (H) from transgenic offspring of founder mouse 13; lactating mammary glands from transgenic offspring of founder mice 19 (I), 33 (J) and 36 (K); testes (L), liver (M), spleen (N) and salivary gland (O) from transgenic offspring of founder mouse 13. Sizes of the protected fragments, calculated from a sequencing ladder, are indicated on the right margin. Location of the 950 b antisense-RNA probe relative to the transgene is indicated below. The probe corresponds to the transcription of the 950 bp *PstI/Hin*dIII fragment. A. 3'-End of the 207 b protected fragment with RNA from day-16 sheep embryos (used as internal standard): this fragment extends from the *PstI* site to the 5'-end of the oTP cDNA, as indicated. B. 3'-End of the 212 b protected fragment with RNA from lactating mammary gland of transgenic offspring of founder mouse 13: this fragment extends from the *PstI* site to the indicated base, determined according to its length.

involved in the process are located at least in one of the following parts of the bαLa gene: the 750 bp 5′ and 450 bp 3′ regions flanking the transcriptional unit, and possibly the second half of the 3′ untranslated region. However, since only one transgenic line expressed the hybrid transgene, a possible influence of the integration site on tissue distribution, although unlikely, cannot be formely ruled out.

All six transgenic lines previously obtained [2] expressed the bαLa transgene, two of them at a high level, whereas three out of four lines carrying the hybrid transgene did not produce any detectable level of oTP mRNA. Such a difference in expression may result from the lack of intron in the hybrid. Similar discrepancies were observed in other experiments involving the WAP gene and intronless derived constructs [14–17]. Furthermore, the importance of introns on the transcriptional efficiency in transgenic mice was recently reported [18,19]. However, the use of sequences derived from an intronless gene was shown to confer an intron-independant expression in vitro [20].

Other factors might affect the level of expression: (i) the integration site, as already observed with the entire bαLa gene [2]; (ii) the occurrence of important *cis*-acting DNA sequences, involved in this regulation, within the transcription unit of the bovine gene; (iii) the 2 bp deletion between the TATA-box and the Cap site in the construct (Figs. 1 and 3), although this does not affect the transcriptional efficiency in vitro [21]; (iv) the occurrence of AU-rich sequences recognized by a cytosolic protein [22] within the 3′-untranslated region of the oTP cDNA, which are responsible, in part, for the susceptibility of many mRNAs to cytoplasmic instability (see [23] for review).

Expression of oTP in line 13 does not apparently affect growth of offspring and fertility of mice. It has been reported that interferon expression in the testes of transgenic mice leads to male sterility [24]. It is possible, but yet to be confirmed, that the sterility of male 43 was due to the expression of the transgene in that tissue.

However, using other constructs derived from the bαLa gene, we have also observed similar Go sterile males (data not shown).

Secretion of miscellaneous foreign proteins, such as human α1-antitrypsin, antihemophilic factor IX, interleukin-2, urokinase, tissue plasminogen activator, CD4 protein and growth hormone, in milk of transgenic animals carrying constructs derived from milk protein-encoding genes have already been reported [15,25-27] (see [3] for review), and the present study, using very short sequences of the bαLa gene, provides another example of the potential of structural elements of milk protein genes to direct expression of hybrid transgenes in the mammary gland. We are currently working on other constructs to try to define more precisely the DNA sequences involved in mammary tissue-specific expression of the bαLa gene and to improve expression of recombinant proteins.

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