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Expression analysis of ruminant α-lactalbumin in transgenic mice: developmental regulation and general location of important *cis*-regulatory elements

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The bovine α-lactalbumin transgene with 750 bp and 336 bp of the 5′ and 3′ flanking region, respectively, is developmentally regulated as its endogenous counterpart in transgenic mice. Comparative expression analysis of three 5′-shortened constructs suggests that the region −477/−220 contains important *cis*-acting transcriptional elements. The level of expression of a long caprine α-lactalbumin transgene encompassing 8.5 kb and 9.5 kb of the 5′ and 3′ flanking region, respectively, was higher but still unrelated to the copy number. Expression of the transgenes and of endogenous milk-protein genes was tissue-specific. In contrast with a recent report, only low amounts of the relevant mRNA were detected in some skin samples, which suggests a possible contamination by mammary tissue.

α-Lactalbumin; Goat; Bovine; Transgenic mouse; Tissue-specificity; Milk composition

1. INTRODUCTION

We have already reported the mammary-specific expression of a bovine α-lactalbumin (bαLa) gene, encompassing 750 bp and 336 bp of the 5′ and 3′ flanking regions, respectively, in transgenic mice [1]. The expression of the transgene, close to that of its endogenous counterpart in the best transgenic lines, was site-dependent and unrelated to the copy number [1]. More recently, it was reported that a guinea-pig αLa transgene was expressed, along with the endogenous β-casein gene, in the sebaceous glands of lactating mice at levels similar to those observed in the mammary gland [2].

In the present study we report the similar developmental expression pattern of the bαLa transgene and its endogenous counterpart, and the levels of expression observed with three 5′-shortened bαLa constructs and a long caprine αLa transgene comprising 8.5 kb and 9.5 kb of the 5′- and 3′-flanking region, respectively [3]. The results suggest that important *cis*-acting transcriptional elements, but not all of them, were present in the investigated bαLa transgene, in particular in the region −477/−220. Furthermore, in contrast with Maschio's report [2], no significant expression of the transgenes and endogenous milk-protein genes was observed in the skin of lactating animals even in the best expressing lines.

Abbreviations: αLa, α-lactalbumin; gαLa, goat α-lactalbumin; bαLa, bovine α-lactalbumin; WAP, whey acidic protein.

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2. MATERIALS AND METHODS

2.1. *Materials*

All enzymes and oligo-labelling kits were purchased from Boehringer Mannheim. [α-^{32}P]dCTP was obtained from Amersham and the RNA purification RNAzol B kit from Bioprobe Systems. Other chemicals came either from Sigma or Poly-labo.

2.2. *Production of transgenic mice*

The vector-free genomic clones (Fig. 1) were micro-injected into the pronuclei of (C57BL/6xCBA) F2 hybrid eggs [4]. Eggs were re-implanted into pseudopregnant recipient mice. The resulting pups were tested for the presence of the transgenes by tail blotting as described in [1].

2.3. *RNA isolation and analysis*

Total RNA was isolated using the RNAzol B kit according to Bioprobe Systems recommendations. Northern analysis was performed as described in [1]. RNA from the mammary gland and the skin of lactating mice from several lines was quantified for the presence of mouse and goat αLa and mouse β-casein, WAP and 28S rRNA transcripts by dot-blotting analysis and scintillation counting of the dot areas of the filters.

2.4. *Milk protein analysis*

Milk samples were collected and defatted as described elsewhere [5]. Western blotting analysis of milk proteins was performed as in [1] using an 'X'-Blot electro-blotting apparatus.

3. RESULTS

3.1. *Generation of transgenic mice*

Ten, six, eleven and four transgenic mice carrying the 21 kb gαLa gene (G) or one of the bovine 5′-shortened E, S and A constructs (Fig. 1), respectively, were obtained. The number of integrated copies of the transgene ranged from 1 to 40, and founder mice S12 and

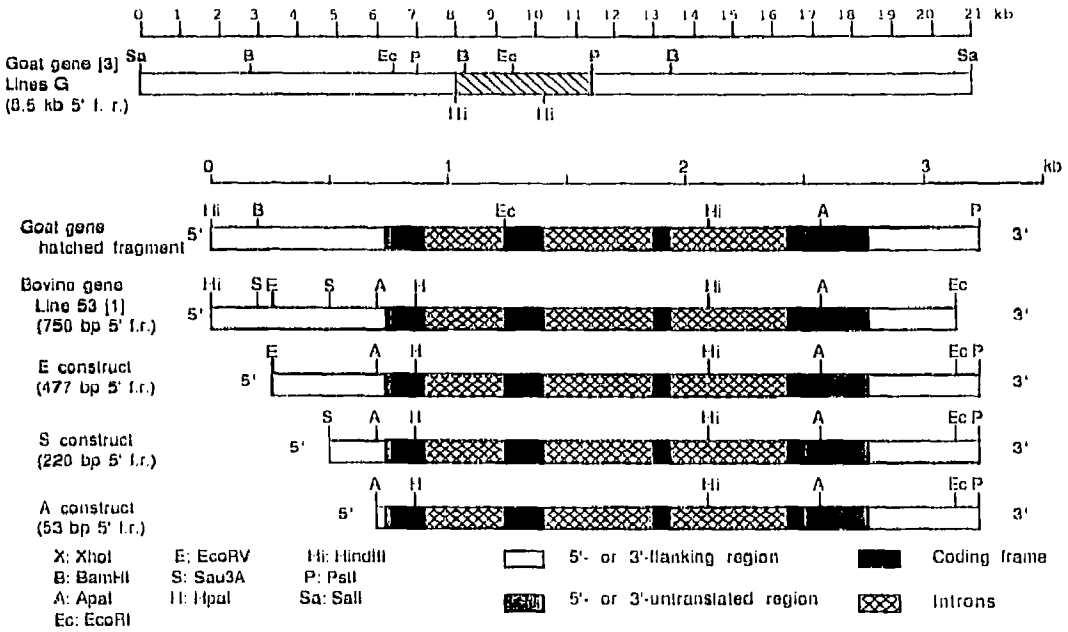


Fig. 1. Structure of αLa transgenes. Top map: structure of the gαLa transgene. The *Hin*dIII-*Pst*I fragment, encompassing the transcription unit of the gene, is hatched. Lower maps: structures of the hatched gαLa fragment and of the bαLa and its 5'-shortened fragments. f.r., flanking region of the transcription unit. Three different bovine genomic libraries were unsuccessfully screened for isolating an αLa gene longer than 3.2 kb; hence the use of the 21 kb caprine αLa gene for the comparative expression studies.

A27 had two integration sites as judged from Southern blotting DNA analysis of their progeny (Table I).

3.2. *Expression of the transgene*

Expression of the transgene was investigated in various tissues (mammary gland, liver, kidney, spleen, salivary gland and skin) of 12-day lactating transgenic descendants of the founder mice by Northern analysis and hybridization with a bαLa cDNA probe [6] (Fig. 2) and RNase protection (data not shown). Transgene transcripts were detected in the mammary gland of all animals from lines G, 53 and E (Table I) but not in those from lines S and A. In general, the expression was mammary-tissue specific, and only low amounts of αLa mRNA were occasionally detected in some tissues as illustrated in Figs. 2 and 3 and summarized in Table I. The transgene was apparently not expressed in various tissues (testis, epididymis, liver, kidney, spleen and salivary gland) of transgenic males from lines G42, G45, E03 and E04 (data not shown).

3.3. *Secretion of exogenous αLa into mouse milk*

Milk samples were collected from transgenic daughters of the founder mouse of each line at day 12 of lactation. Presence of exogenous αLa was assessed by Western-blotting analysis and its concentration estimated using internal standards of purified αLa (Fig. 4 and Table I). It ranged from undetectable levels in several lines (less than 0.001 mg/ml) up to 0.1 mg/ml, 1.2 mg/ml and 3.7 mg/ml in lines E04, G45 and G42, respectively.

3.4. *Developmental regulation during gestation of bαLa*

Developmental regulation of the bαLa gene with 750 bp and 53 bp of 5' and 3' flanking regions, respectively, was investigated in line 53 [1]: dot blot analysis was performed on RNA samples isolated from mammary and liver tissues of transgenic descendants of founder mouse 53 at various stages of pregnancy and lactation (Fig. 5). Expression of the transgene appears to be turned on between day 15 and day 18 of pregnancy. Similar results were obtained when these RNA samples were probed with mouse αLa cDNA ([7], data not shown).

4. DISCUSSION

Expression analysis of the investigated transgenes suggests that only 477 bp of the 5'-flanking region is sufficient for the tissue-specific expression of bαLa, compared to the 750 bp used in our previous report [1], and that important *cis*-acting elements involved at least

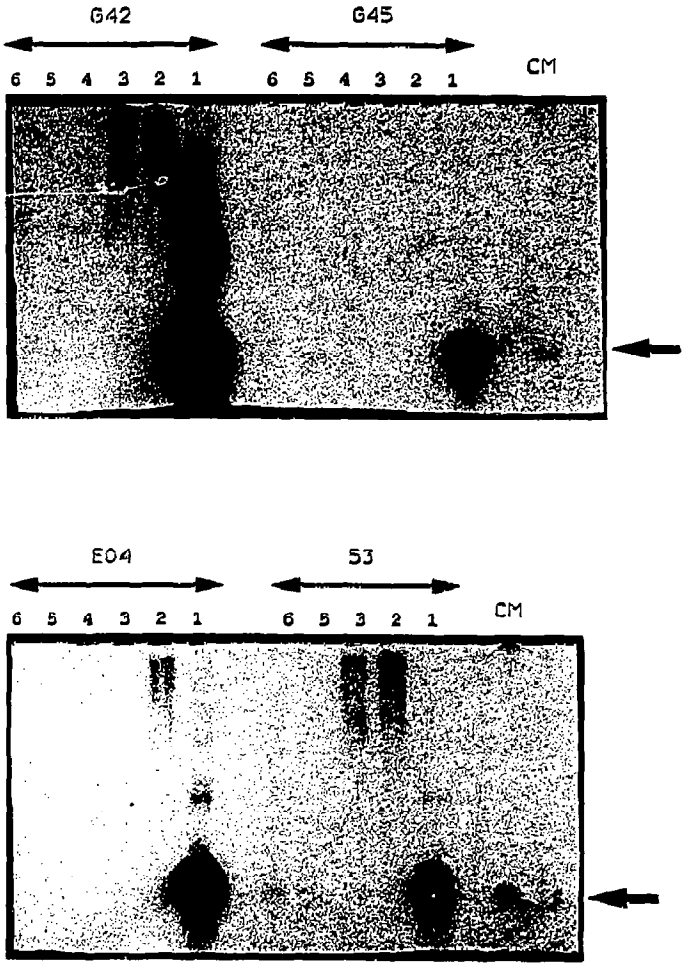


Fig. 2. Tissue-specific expression of αLa transgenes. Northern blot analysis was performed using 10 μg of total RNA from 12 day lactating descendants of founder mice G42, G45, E04 and 53. Tissues analyzed were mammary gland (1), liver (2), kidney (3), spleen (4), salivary gland (5) and skin (6). Lane CM, 10 μg of total RNA from 12 day lactating mammary gland of non-transgenic mouse. The faint band detected arose from a slight cross-hybridization between murine αLa mRNA and the bαLa cDNA probe. The blots were probed with an oligo-labelled bαLa cDNA [6]. Arrows indicate the position of mature αLa mRNA. Higher molecular weight bands observed in the mammary gland lanes might represent RNA precursors. The smear observed in lanes 2 and 3 probably represents DNA.

in the regulation of its transcriptional level are located within a 257 bp fragment (position −477 to −220) as no detectable expression was observed with constructs carrying 220 bp or less of the 5′-flanking region.

The content of αLa ranged from undetectable levels up to 1.2 and 3.7 mg/ml of milk in transgenic lines carrying the 21 kb gαLa, to be compared with the endogenous αLa content of mouse's (0.8 mg/ml, [8]) and goat's (2 mg/ml, [9]) milk. However, the expression was still site-dependent and copy-number unrelated, suggesting that some *cis*-regulatory elements are lacking.

The weak expression occasionally detected in the salivary glands, kidney, or spleen of lactating animals of five lines (Fig. 2 and Table I) has also been reported for other milk protein-encoding genes [1,2,5,10–13] and such a 'leakage' might be ascribed to an integration site-position effect [14].

The endogenous mouse β-casein and a guinea-pig αLa transgene were recently reported to be expressed in the skin of lactating mice, at levels similar to that found in the mammary gland [2]. In contrast, we did not find any detectable level of expression of the endogenous β-casein, WAP, αLa genes and αLa transgenes in the skin of 3 or 12 day lactating non-transgenic mice and of all but three transgenic lines analyzed (Fig. 3). Similarly, we did not observe any expression of endogenous

Table I

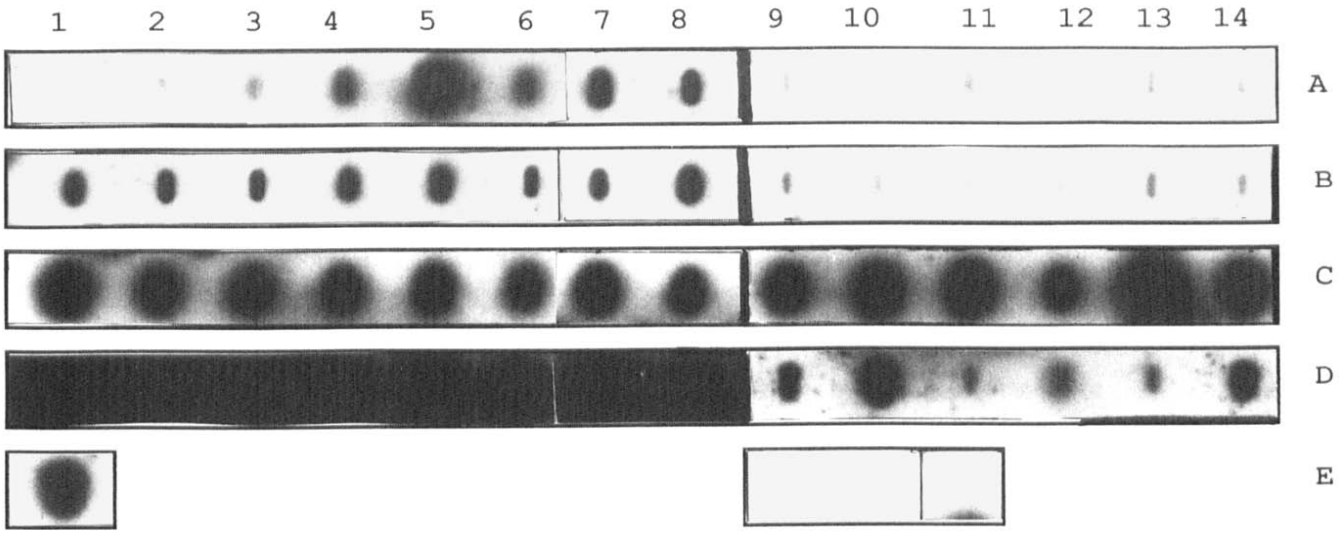
Summary of αLa expression in the different lines

Results obtained with the 21 kb gαLa transgene (G) are written in italics. E, S, and A refer to the 5'-shortened bαLa constructs (Fig. 1). RNA amounts are expressed relative to the level of αLa mRNA in bovine lactating mammary gland. N.D., not determined; N, non-detected. Tissues in which a very weak expression of the transgene was detected are indicated. S.G., salivary gland; Sk, skin; G0, founder mouse; G1, progeny (first generation) of founder mice S12 and A27 which transmitted two independent integration sites.

Line	Copies integrated G0 G1	Germline transmission	Number of integration site	Expression in transgenic offspring		
				mRNA/Tissue specificity	Relative amount (%)	Protein in the milk (mg/ml)
G04	*05*	*6/17 (35%)*	*1*	*Yes/Yes*	*N.D.*	*<0.001*
G05	*10*	*4/17 (23.5%)*	*1*	*Yes/kidney*	*N.D.*	*<0.001*
G13	*20*	*2/ 9 (22%)*	*1*	*Yes/Yes*	*40%*	*<0.25*
G14	*10*					
G15	*05*	*6/15 (40%)*	*1*	*Yes/Yes*	*N.D.*	*<0.001*
G25	*05*	*5/ 9 (55.5%)*	*1*	*Yes/Yes*	*N.D.*	*<0.001*
G34	*05*	*4/16 (25%)*	*1*	*Yes/Yes*	*70%*	*0.4*
G42	*05*	*6/24 (25%)*	*1*	*Yes/S.G., Sk?*	*700%*	*3.7*
G45	*01*	*7/21 (33%)*	*1*	*Yes/S.G.*	*180%*	*1.2*
G52	*10*	*4/12 (33%)*	*1*	*Yes/Yes*	*N.D.*	*0.002*
53 [1]	02	5/30 (17%)	1	Yes/S.G., Sk?	50%	0.45
E02	05	4/16 (25%)	1	Yes/Yes	N.D.	<0.001
E03	10	7/18 (39%)	1	Yes/Yes	20%	0.075
E04	15	1/25 (4%)	1	Yes/Spleen, Sk?	110%	0.1
E06	05	0/15				
E12	02	0/29				
E14	01	0/19				
S02		Sterile				
S12	40 35	8/24 (33%)	2	N		<0.001
	05			N		<0.001
S13	05	2/18 (11%)	1	N		<0.001
S17	05	5/13 (38.5%)	1	N		<0.001
S18	05	3/12 (25%)	1	N		<0.001
S19	30	8/11 (72%)	1	N		<0.001
S20	40	1/18 (5.5%)	1	N		<0.001
S24	05	4/ 9 (44.5%)	1	N		<0.001
S36	05	3/ 6 (50%)	1	N		<0.001
S40	10	1/ 7 (14%)	1	N		<0.001
S42	30	5/12 (41%)	1	N		<0.001
A22	10	4/6 (67%)	1	N		<0.001
A27	03 02	6/19 (31%)	2	N		<0.001
	01			N		<0.001
A37	05	3/6 (50%)	1	N		<0.001
A43		Sterile				

β-casein and αLa genes in the skin of lactating goats (data not shown). The very low level of gαLa or bαLa transcripts detected in the skin of lactating mice from lines G42, 53 and E04, together with minute amounts of endogenous WAP, αLa and β-casein transcripts (Fig. 3), might result from a contamination of the samples by mammary epithelial cells, assuming that the WAP gene is not expressed in the skin [10]. This is not unlikely as the mammary system extends through the body of lactating mice [15]. The alternative is an individual variation in the expression pattern of milk-protein genes [16]. Further experiments will be needed to explain the discrepancy between our results and those of Maschio et al. [2].

The bαLa transgene and its endogenous counterpart shared the same developmental pattern, as the induction of their expression was observed between day 15 and 18 of pregnancy, in accordance with previous data [7].



MOUSE	NON TRANSGENIC 03 DAYS LACT.		NON TRANSGENIC 12 DAYS LACT.		LINE G42 12 DAYS LACT.		LINE G45 12 DAYS LACT.		LINE 53 12 DAYS LACT.		LINE E04 12 DAYS LACT.	
TISSUE / PROBE	M.G.	SKIN	M.G.	SKIN	M.G.	SKIN	M.G.	SKIN	M.G.	SKIN	M.G.	SKIN
BOV. αLA	N.S.	N.S.	N.S.	N.S.	3910	98 (2.5%)	960	N.S.	250	N.S.	700	N.S.
Mouse αLA	587	N.S.	411	N.S.	460	N.S.	601	N.S.	418	N.S.	530	N.S.
Mouse WAP	8952	N.S.	11334	659 (5.8%)	9166	286 (3.1%)	14100	505 (3.5%)	11220	366 (3.3%)	11600	383 (3.3%)

Fig. 3. RNA quantification. Lanes 1 to 8, 5 μg of total RNA from the mammary gland of: 1 and 2, 12 and 3 day lactating non-transgenic mice, respectively; 3 to 8, 12 day lactating transgenic descendants of founder mice G13, G34, G42, G45, 53 and E04, respectively. Lanes 9 to 14, 10 μg of total RNA from the skin of: 9 and 10, 12 and 3 days lactating non-transgenic mouse, respectively; 11 to 14, 12 days lactating transgenic offsprings of founder mice G42, G45, 53 and E04, respectively. Probes are indicated on the right margin: A, bαLa cDNA [6]; B, mouse αLa cDNA [7]; C, 28S rRNA oligonucleotide [18]; D, mouse WAP cDNA [19]; E, mouse β-casein cDNA [20]. Lower table: scintillation counting of the dot areas of the filters. The Northern blot was normalized with the 28S rRNA probe. The percentage of expression in the skin, relative to that in the mammary gland (M.G.), is indicated. N.S., non-significant count relatively to the background. LACT., lactation.

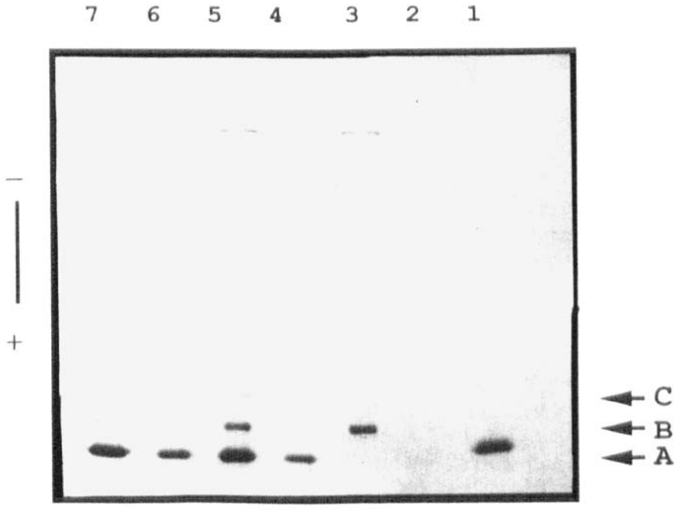


Fig. 4. Western blot analysis of milk proteins. Lane 1, 100 ng of gαLa; lane 2 and 3, 0.04 and 0.18 μl of milk from 12 day lactating non-transgenic mouse; lane 4 and 5, 0.04 and 0.18 μl of milk from 12 day lactating transgenic descendant of mouse G45; lane 6 and 7, 0.02 and 0.09 μl of milk from 12 day lactating transgenic descendant of mouse G42. Right margin: A, major form of gαLa protein; B, detected endogenous protein which might correspond to mouse αLa; C, minor glycosylated (?) form of gαLa protein, as already described in goat's milk [9].

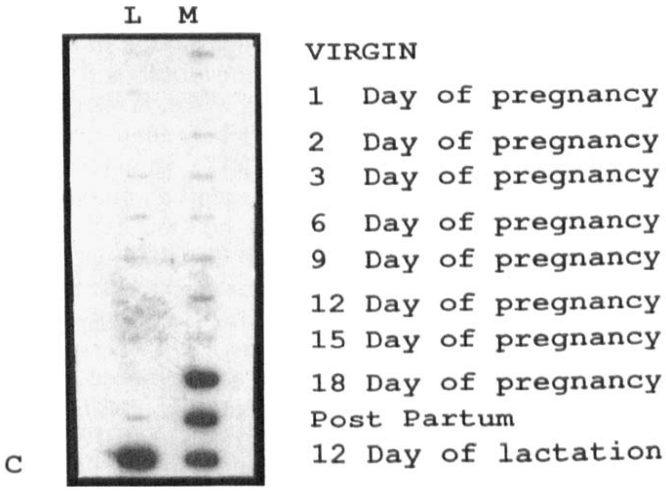


Fig. 5. Developmental regulation of the bαLa transgene in line 53. Each dot corresponds to 5 μg of total RNA isolated, as indicated on the right margin, from the mammary gland (lane M) or from the liver (lane L) of transgenic descendants of founder mouse 53, at different stages of pregnancy. Dot C, 5 μg of total RNA from lactating cow mammary gland. Hybridization was performed with bαLa cDNA [6].

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