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A 17.6 kbp region located upstream of the rabbit WAP gene directs high level expression of a functional human protein variant in transgenic mouse milk

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We have investigated whether DNA regions present in the rabbit whey acidic protein (WAP) promoter/5′ flanking sequence could potentially confer, in vivo, high level expression of reporter genes. Transgenic mice were generated expressing a variant of human α_1-antitrypsin, which has inhibitory activity against plasma kallikrein under the control of a 17.6 kbp DNA fragment located upstream of the rabbit WAP gene. Up to 10 mg/ml of active and correctly processed recombinant protein were detected in mouse milk, thus suggesting that the far upstream DNA sequences from the rabbit WAP gene might be useful for engineering efficient protein production in the mammary glands of transgenic animals.

Rabbit; WAP gene; α_1-Antitrypsin; Transgenic mouse; Milk; Plasma kallikrein

1. INTRODUCTION

Whey acidic protein (WAP) is the principal whey protein found in mouse, rat and rabbit milk [1–4]. WAP synthesis is complex, being regulated by glucocorticoids and prolactin as well as by cell–cell and cell–extracellular matrix interactions, thus indicating that efficient expression is achieved only when a correctly organised cellular structure similar to that found in the mammary gland is maintained [5]. The promoter and upstream sequences (2.6 kbp) of the murine WAP gene have been used to direct the tissue and developmental expression of several foreign proteins in the milk of transgenic mice [6–9], the expression levels of the chimeric transgenes being relatively low with respect to those of endogenous milk proteins or to exogeneous transgenic mouse or rat WAP [10–12]. This suggests that regulatory DNA sequences not included in the transgenic construct might play a role in order to attain high levels of expression of the exogenous gene.

Previous in vitro studies [13] had identified important regulatory elements within a 6.3 kbp fragment located upstream of the rabbit WAP gene. Here we report the generation of transgenic mice containing a fusion transgene in which the gene coding for a modified version of human α_1-antitrypsin (α_1AT) was ligated to a 17.6 kbp fragment of the rabbit 5′ flanking region WAP gene.

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2. MATERIALS AND METHODS

2.1. *Materials*

In the ELISA assay goat anti-human α1AT antisera and peroxidase-linked anti-goat antibodies were from Cappel (Malvern, PA, USA). Human plasma α_1AT was obtained from Calbiochem (Meudon, France). RID plates were purchased from Behring (Marburg, Germany). Human plasma kallikrein and the chromogenic substrate, S-2302, were from Kabi Vitrum (Stockholm, Sweden). Peroxidase-coupled immunoglobulin G fraction of goat anti-human-α_1AT antibodies was obtained from Cappel. All other reagents were of analytical grade.

2.2. *DNA construct*

The genomic sequences corresponding to the human α_1AT gene and the rabbit WAP promoter region [14] were isolated as separate lambda EMBL3 or Charon 4A clones from a human lymphocyte DNA library [15] and from a rabbit liver library (prepared by T. Maniatis), respectively. Using site-directed mutagenesis amino acid residues 357 (Pro) and 358 (Met) of human α_1AT were changed into Ala and Arg residues, respectively, thereby generating the genomic α_1AT (Ala-357, Arg-358) variant [16,17] which was subsequently introduced downstream of a 17.6 kbp rabbit WAP promoter fragment (engineered *Hin*dIII site [13,14]) cloned in the vector pPolyIII-I* [18]. The resulting plasmid was named pTG1606, and the transgene (Fig. 1) could be isolated from the procaryotic sequences by a *Not*I restriction digest.

2.3. *Generation of transgenic mice*

Transgenic mice were generated according to [19] using C57Bl/6xSJL hybrid crosses and identified by tail DNA analysis on Southern blots.

2.4. *Concentration of α_1AT (Ala-357, Arg-358) in lactoserum and blood serum*

Milk samples were collected at day 13 after parturition from anesthesized mice previously injected with 0.05 U of oxytocin to stimulate milk let-down [20]. Mouse milk was diluted (1:5) in distilled water, fat was removed after centrifugation and aliquots were stored frozen at

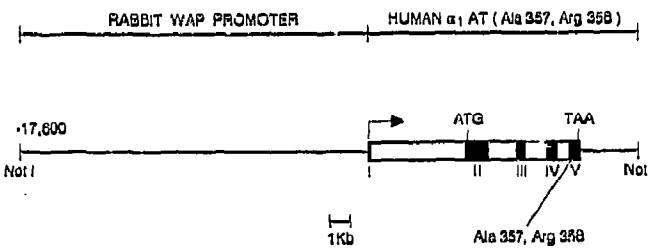

Fig. 1. Schematic representation of the injected fusion transgene derived from pTG1606 after digestion with *Not*I. Filled and open boxes represent exons (roman numbers) and introns of the variant α_1AT gene, respectively. Solid line indicates 5′ flanking regions (up to −17,600 bp from the CAP site, arrowed) of the rabbit WAP gene as well as 3′ flanking regions of the human α_1AT gene (approximately 3,000 bp).

−70°C until use. Blood was collected from tails, allowed to coagulate at room temperature and the serum stored frozen at −70°C until use. The quantification of α_1AT (Ala-357, Arg-358) was done by ELISA [21].

2.5. *Electrophoretic profile*

SDS-PAGE was performed using 10% (w/v) acrylamide [22]. Protein bands were stained with Coomassie blue R-250 and immunoblotting was performed according to [23]. Detection was performed using horseradish peroxidase and 4-chloro-1-naphtol.

2.6. *Functional activity assay*

Anti-kallikrein activity was measured as described [17] at a human plasma kallikrein concentration of 7 nM. α_1AT (Ala-357, Arg-358), either purified from *E. coli* or present in mouse lactoserum, was added to the reaction mixture at a concentration of 39.1 or 59.8 nM, respectively. The reaction was started by adding the chromogenic substrate, S-2302, to a final concentration of 1.7 mM. α_1AT (Ala-357, Arg-358) concentrations were determined by radial immunodiffusion (RID) according to the manufacturers recommendations. Measurements were done on a 8451 diode array spectrophotometer (Hewlett-Packard, Waldbronn, FRG). Second order association constants k_{ass} were determined from the measured pseudo-first order rate constants, k', as described [17] using a minimal excess of five-fold of inhibitor over protease. Data were analyzed by linear regression of the semi-logarithmic plot (correlation coefficients between 0.994 and 0.997).

3. RESULTS

3.1. *Expression of transgenic α_1AT (Ala-357, Arg-358)*

The transgene construct includes 17.6 kbp of the rabbit WAP promoter with the transcription start site fused to the gene coding for the (Ala-357, Arg-358) variant of the human α_1AT (Fig. 1) in which the substrate specificity of α_1-antitrypsin was changed from neutrophil elastase to plasma kallikrein [16].

Nine transgenic founders were identified (5 females and 4 males). A high degree of cannibalism of the mothers towards their litters was observed. Eventually four out of five mothers were successfully capable of lactating their pups. α_1AT (Ala-357, Arg-358) antigen levels were measured in both lactosera and blood sera at day 11–13 after litter birth (Table I), showing elevated secretion of the human protein in both fluids (up to 6 mg/ml and 700 μg/ml in milk and serum, respectively, for female 105). No direct correlation between copy number of the integrated transgene and α_1AT (Ala-357, Arg-358) levels could be made (not shown). Moreover blood serum levels ranging up to 35 μg/ml were found in transgenic males.

Transgenic male 106 produced eight F1 transgenics of which two were females (106 I 42 and 106 I 45). α_1AT (Ala-357, Arg-358) levels in lactoserum and blood serum reached up to 10.5 mg/ml and 220 μg/ml, respectively. Levels in male descendants were similar to those of the founder, 106. A time–course of α_1AT (Ala-357, Arg-358) levels in blood serum from two females (not shown) demonstrated basal concentrations of around 60–80 μg/ml with peaks (210–230 μg/ml) at day 18 after parturition and a slow decrease to normal levels after 113 days post litter birth.

3.2. *Biochemical characterization of α_1AT (Ala-357, Arg-358)*

α_1AT (Ala-357, Arg-358) secreted into the milk of transgenic mouse 106 I 45 was first analyzed by SDS-

Table I

Antigen levels of α_1AT (Ala-357, Arg-358) (μg/ml) in lactosera and blood sera obtained from F0 and F1 transgenic donors

F0	Lacroserum	Blood serum	F1	Lactoserum	Blood serum
44 ♀	6,000	310	106 I 42 ♀	9,250	150
77 ♀	370	ND	106 I 45 ♀	7,000–10,500	150–220
105 ♀	6,000	700	106 I 47 ♂		20
112 ♀	1,400	23	106 I 49 ♂		13.5
124 ♀	NDD	NDD			
106 ♂		35			
12 ♂		22			
135 ♂		14			
133 ♂		ND			

Samples were collected between day 11–13 postpartum after oxytocin injection. Values from mouse 106 I 45 are representative of two samplings. Antigen levels in control normal mice were ≤10 ng/ml. ND, not detectable; NDD, not determined (mouse 124 failed repeatedly to give birth or else cannibalised her litter).

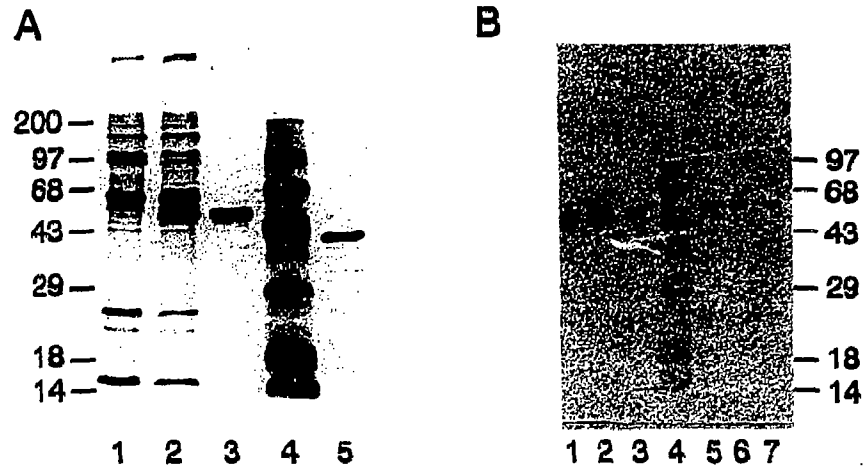

Fig. 2. (A) SDS-PAGE under denaturing and reducing conditions of lactoserum from a normal mouse (lane 1), lactoserum from transgenic mouse 106 I 45 (lane 2), purified α_1AT from human plasma (lane 3), molecular weight markers (lane 4) and purified α_1AT (Ala-357, Arg-358) from a recombinant strain of *E. coli* (lane 5). (B) Immunoblot after SDS-PAGE of lactoserum from transgenic mouse 106 I 45 (lanes 1–3; 200, 100 and 50 ng of α_1AT (Ala-357, Arg-358) as determined by RID) and of α_1AT purified from human plasma (lanes 5–7; 200, 150 and 100 ng by RID). Lactoserum from control normal mice was negative. Molecular weight markers in kDa are indicated on the figure margins.

PAGE under denaturing and reducing conditions, which showed that it was the major protein in lactoserum (Fig. 2A, lane 2). The recombinant molecule had electrophoretic mobility identical to α_1AT isolated from human plasma (Fig. 2A, lane 3), indicating that it was glycosylated. α_1AT (Ala-357, Arg-358) purified from a recombinant strain of *E. coli* which was lacking carbohydrates [17] had a faster electrophoretic profile (Fig. 2A, lane 5). Scanning of lane 2 (Fig. 2A) of the Coomassie blue-stained gel showed that approximately 35% of all the proteins in lactoserum of the transgenic mouse corresponded to the recombinant molecule. Lactoserum from the transgenic mouse was subsequently analyzed by immunoblotting after SDS-PAGE and compared to α_1AT from human plasma (Fig. 2B). A major immunoreactive band migrating with an apparent molecular weight identical to that of the human molecule was present. However, in lactoserum a second protein band with slightly reduced electrophoretic mobility stained less intensely (Fig. 2B, lanes 1–3). Lactoserum from the milk of normal mice did not respond upon immunoblotting (not shown).

Inhibitory activity of α_1AT (Ala-357, Arg-358) in lactoserum from mouse 106 I 45 was determined by measuring the second order association rate constant (k_{ass}) with human plasma kallikrein in comparison to the purified inhibitor obtained from *E. coli*. Lactoserum from normal mice served as a negative control. Table II summarizes the results showing that α_1AT (Ala-357, Arg-358) in lactoserum of the transgenic mouse or purified from recombinant *E. coli* had identical association rate constants with human plasma kallikrein, thus corresponding well with previously determined values [17]. Lactoserum from normal mice did not show any inhibition of plasma kallikrein in this assay.

Table II

Second order association rate constants in $M^{-1} \times s^{-1}$

Lactoserum (mouse 106 I 45)	1.64×10^5
α_1AT (Ala-357, Arg-358) (*E. coli*)	1.71×10^5
Lactoserum (normal mouse)	no inhibition

4. DISCUSSION

This study represents a first step towards the understanding of the complex regulation of the rabbit WAP promoter in vivo.

Our data suggest that DNA sequences located up to 17.6 kbp upstream of the rabbit WAP gene are responsible for the high levels of reporter human protein present in the milk of transgenic mice. The promoter/5′ flanking sequence employed herein contains hormone-responsive elements which were previously characterized using rabbit primary mammary cells [13] and which might be responsible for such elevate levels. However, further upstream regions (between −17.6 and −6.3 kbp) may be implicated. Alternatively, the particular human gene employed here or regulation at the post-transcriptional level might be responsible for such elevate expression. We are at present investigating these hypotheses.

High levels of α_1AT (Ala-357, Arg-358) were found in blood serum of transgenic females and males, being in direct correlation with the lactating period in the females. It is conceivable that the regulatory DNA sequences present in this particular construct confer tissue-specificity which is not restricted to the mammary gland alone. Preliminary data on WAP-regulated trans-

gene expression support this concept. In any case such high circulating levels might result in toxicity and account therefore for the observed cannibalism.

Recombinant α_1AT (Ala-357, Arg-358) comprised about 35% of total protein in mouse lactoserum underscoring its efficient expression and secretion and proved to be as efficient in inhibition of human plasma kallikrein as the unglycosylated counterpart purified from a recombinant strain of *E. coli*. A minor band recognised as the α_1AT variant migrated with a lower apparent molecular weight in lactoserum of a transgenic mouse. This might correspond to a degradation product of the recombinant protein presumably through cleavage in its exposed inhibitory loop or to a different glycosylated form. A similar observation was also reported in the case of human α_1AT expressed in transgenic mouse milk [24] and may raise important questions concerning both stability (e.g. temperature, presence of proteases) and post-translational microheterogeneity of recombinant proteins in the milk of transgenic animals.

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