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High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C

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ABSTRACT **Transgenic pigs were generated that produced human protein C in their milk at up to 1 g/liter. The gene construct was a fusion gene consisting of the cDNA for human protein C inserted into the first exon of the mouse whey acidic protein gene. These results demonstrate that the mouse whey acidic protein gene contains regulatory elements that can direct cDNA expression at high levels in the pig mammary gland. Recombinant human protein C that was produced at about 380 μg/ml per hr in transgenic pig milk possessed anticoagulant activity that was equivalent to that of protein C derived from human plasma. These studies provide evidence that γ-carboxylation can occur at high levels in the mammary gland of a pig.**

Several different regulatory sequences have been identified for milk protein genes that enable the expression of heterologous proteins in the milk of transgenic animals (1–4). However, expression levels from cDNAs (1, 4) and genomic sequences have been variable, with genomic sequences frequently producing much higher levels of protein (2, 3). For example, the cDNA encoding human factor IX (hFIX) was expressed in sheep milk at only 25 ng/ml using a fusion gene consisting of 4.0 kilobase pairs (kbp) of 5′ flanking sequence from the sheep β-lactoglobulin gene (BLG), 1.5 kbp of hFIX cDNA, and 4.9 kbp containing the BLG transcription unit and 3′ flanking sequence (1). In contrast, secretion of up to 30 mg/ml of human α_1-antitrypsin (hAAT) into sheep milk was achieved using the same 4.0 kbp of BLG 5′ flanking sequence fused to 6.5 kbp of hAAT minigene (first intron removed) coding sequence (3). Additionally, there is no direct correlation between the level of expression in transgenic mice compared to livestock for a given genetic construct (2, 4). For example, transgenic pigs expressed a 7.2-kbp genomic fragment of mouse whey acidic protein (WAP) at a 2- to 100-fold greater level (2, 5) than transgenic mice with the same construct. Therefore, the choice of employing a cDNA versus a genomic construct to synthesize a given protein in the mammary gland of livestock can become complex.

Human protein C (hPC) is a regulator of hemostasis, suggesting its potential use as a therapy for many disease states (6). Protein C is a zymogen of a serine protease that is activated by thrombin (6). The structure of hPC is complex and its level of expression in recombinant mammalian cells (7) and transgenic mice (8) has been limited to <1 μg/ml per hr. In this report, we detail the expression of functional recombinant hPC (rhPC) in the milk of transgenic swine at 1000 μg/ml per hr using a hybrid genetic construct consisting of the cDNA of hPC regulated by the mouse WAP gene.

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MATERIALS AND METHODS

Transgenic Swine. Crossbred gilts served as embryo donors and recipients of microinjected eggs. Estrus synchronization, surgical procedures, and pronuclear microinjection of centrifuged zygotes were performed as described by Wall *et al.* (9). About 1–3 pl of DNA solution (3.3 μg of DNA per ml in 10 mM Tris·HCl/0.25 mM EDTA, pH 7.4) was microinjected as described by Brinster *et al.* (10). The hybrid transgene (WAPPC-1) consists of the cDNA for hPC inserted into the unique *Kpn* I site in the first exon of the mouse WAP gene (11), as illustrated in Fig. 1.

Tail tissue was biopsied from 2-day-old piglets and DNA was isolated using a modification of the procedure developed by Marmur (12). Transgenic founder animals were identified initially by polymerase chain reaction (PCR) (13) using hPC-specific primers and later confirmed by Southern analysis using ^{32}P-labeled hPC cDNA.

Mammary gland biopsies were performed on two transgenic females and a control female on days 55 and 35 of lactation, respectively. Biopsies were washed briefly in sterile saline and immediately frozen in liquid nitrogen. Total RNA was isolated from the pig mammary gland biopsies and human liver tissue samples (14). RNA samples (10 μg) were fractionated using agarose/formaldehyde gels and then transferred by vacuum onto nylon membranes. The membranes were probed with ^{32}P random primer-labeled hPC cDNA or WAP cDNA.

Pig Milk Collection and Preparation. Piglets were removed from the sows for ≈30 min prior to milking to allow for milk accumulation. Milk letdown was induced by intramuscular administration of 20–30 international units of oxytocin. Milk was collected directly into Tris-buffered saline/EDTA buffer (2× TBS/EDTA: 100 mM Tris·HCl, 300 mM NaCl, 200 mM EDTA, pH 6.5, chilled to 0–2°C) in a 1:1 buffer to milk ratio. Fat and precipitate were removed by centrifugation at 15,000 × *g* for 20 min at 0–2°C. The diluted whey was then filtered through sterile gauze to remove residual solids and stored at −90°C. Control pig milk was treated identically.

Analysis of rhPC. Expression levels in the whey were determined by ELISA, using a monoclonal antibody (HPC4-Mab) that has a calcium-dependent binding epitope in the activation peptide (15). The rhPC captured by the HPC4-Mab was detected using horseradish peroxidase conjugated to goat anti-rabbit IgG following a 3-hr incubation at room temperature with a rabbit polyclonal antibody to hPC. A second ELISA procedure was also used in which rabbit polyclonal anti-hPC antiserum was used to immunocapture

Abbreviations: hPC, human protein C; rhPC, recombinant hPC; WAP, whey acidic protein; nt, nucleotide(s); APTT, activated partial thromboplastin time; NRPP, normal reference plasma pool.

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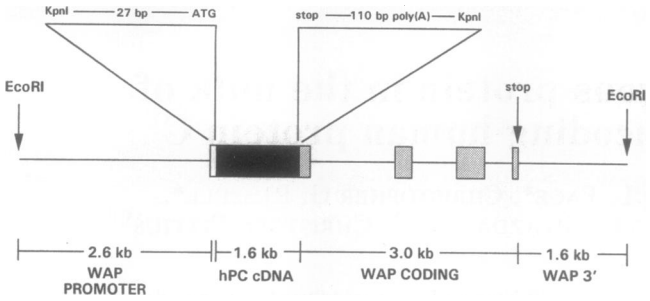

FIG. 1. Diagram of the WAPPC-1 construct. The 1.6-kbp cDNA for hPC [including 110 bases of poly(A)] was inserted at the *Kpn* I site at the first exon of WAP, using *Kpn* I linker DNA. The genomic WAP gene consisted of 2.6 kbp of 5′ flanking promoter sequence, 3.0 kbp of coding sequence (exons and introns), and 1.6 kbp of 3′ flanking DNA. Noncoding DNA segments and introns are indicated by dark lines. The open box is the linker DNA, the filled box is the cDNA for hPC, and stippled boxes are WAP exons. Plasmid DNA was isolated, digested with *Eco*RI, and purified using HPLC to remove all traces of cloning vector DNA (8).

rhPC followed by detection with a sandwich of goat anti-hPC antiserum and rabbit anti-goat antiserum conjugated to horseradish peroxidase.

The rhPC from transgenic pig whey was purified and fractionated by using three successive immunosorptions by HPC4-Mab immobilized onto Affi-Prep 10 (BioRad). Briefly, the whey was diluted to 15 mg of total protein per ml and then loaded onto the column in the presence of 50 mM Ca^{2+} in TBS (pH 7.2) at 3-fold excess of column capacity assuming 0.2 mg of hPC antigen per ml of immunosorbent. The rhPC that was bound to the immunosorbent for each of three successive immunodepletions of the starting whey was specifically eluted with 50 mM EDTA in TBS (pH 7.2). The fall-through from the first immunopurification was applied to the same immunosorbent but at 10% of column capacity based upon antigen levels detected by polyclonal ELISA. The fall-through of the second immunopurification was adjusted to pH 8.5 and applied to the HPC4-Mab immunosorbent at 10% of column capacity based upon antigen levels detected by polyclonal ELISA.

The biological activity of the rhPC was measured using an activated partial thromboplastin time (APTT) assay (16). The APTT reagent included Protac (*Agkistrodon contortrix* venom, American Diagnostica, Greenwich, CT) to specifically activate hPC or rhPC prior to adding $CaCl_2$ to initiate coagulation.

RESULTS

DNA Analysis. A total of 26 piglets were born from 8 recipients that had received embryos microinjected with the WAPPC-1 construct. Screening of genomic DNA from tail biopsies using the PCR indicated that 7 piglets contained the transgene, for an integration frequency of 27%. Southern analysis of DNA from these pigs (Fig. 2*A*) identified only 5 positive for the transgene, including a male that died shortly after birth (not shown in Fig. 2*A*). Assuming hemizygosity, female founder animals 29-2, 83-1, 83-2, and 83-3 were estimated (by PCR of serial DNA dilutions) to have 10, 20, 5, and 1 copy of the transgene per genome, respectively (Table 1). Two other founder gilts (29-1 and 83-6) appeared to be mosaic.

Southern blot analysis of tail DNA (digested with *Eco*RI, which excises the entire WAPPC-1 transgene) from several founder pigs is shown in Fig. 2*A*. Up to three bands hybridized with the hPC cDNA probe (lanes 6 and 7) and were consistent with sizes of monomers, dimers, and trimers of the transgene. DNA from other founder animals exhibited a

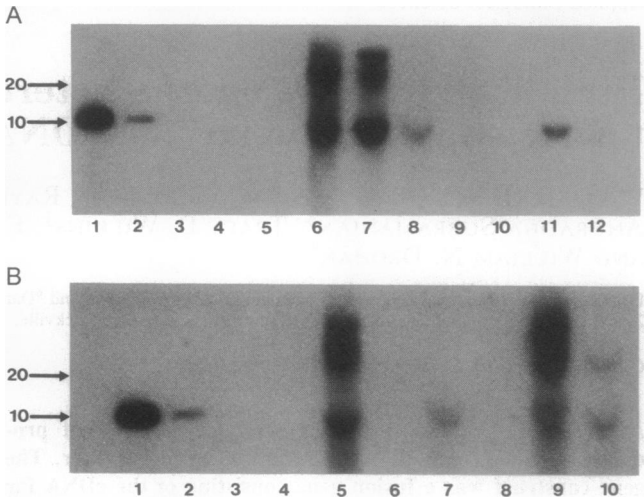

FIG. 2. Southern analysis of transgenic swine. DNA was isolated from tail biopsies of 2-day-old piglets. DNA samples were digested with *Eco*RI, run on 0.7% agarose gels, transferred to nitrocellulose membranes, and hybridized with ^{32}P random primer-labeled hPC cDNA. Arrows indicate sizes of DNA bands in kbp. (*A*) Founder animals. Lanes: 1–3, 250, 25, and 2.5 pg of *Eco*RI-digested WAPPC-1 plasmid, respectively; 4–12, 5 μg of *Eco*RI-digested DNA from control pig and founder pigs 29-1, 29-2, 83-1, 83-2, 83-3, 83-4, 83-5, and 83-6, respectively. (*B*) Pig 29-2 and offspring. Lanes: 1–4, same as in *A*; 5, founder pig 29-2; 6–10, offspring from founder animal 29-2.

single band at about 9 kbp (lanes 8 and 11). Southern analysis of DNA from founder pig 29-2 and her offspring is presented in Fig. 2*B*; three of five piglets contained the transgene. Overall germ-line transmission of the transgene is summarized in Table 1. Only one of the six founder females (83-6) failed to transmit the transgene. Among 53 offspring from transgenic founder animals, 28 contained the transgene, for an overall frequency of germ-line transmission of 53%.

RNA Analysis. Northern blots of normal human liver RNA and mammary gland RNA from pigs 29-1, 29-2, and control were probed with ^{32}P random primer-labeled hPC cDNA (Fig. 3*A*). Human liver RNA (lanes 1, 2, 6, and 7) exhibited a single weak band at about 1600 nucleotides (nt). A very strong band of about 1480 nt was detected in RNA from pig 29-2 (lane 5) but not in RNA from the control pig (lane 3) or transgenic pig 29-1 (lane 4). Additional weak bands of approximately 2360, 2630, and 4320 nt were detected in the RNA of pig 29-2 (lane 5) but not in the other pig RNA samples nor the human RNA samples. Similar amounts of total RNA (from both tissue types) were loaded onto the gel based on 18S and 28S rRNA band intensities. The hPC transcript

Table 1. Detection of transgenic founder animals, offspring, and rhPC expression

Pig no.*	Gene copy no.†	Germ-line transmission‡	hPC antigen, μg/ml	
			By HPC4-Mab ELISA	By polyclonal ELISA
29-1	≪1	6/9	2–3§	1–3§
29-2	10	4/7	10–420§	200–1000§
83-1	20	3/6	50–260§	140–650§
83-2	5	9/12	ND	4–8
83-3	1	6/7	ND	ND
83-6	≪1	0/12	0.5–1.0	2–5

ND, none detected.
*One transgenic male founder pig not included.
†Transgene copy number estimated by PCR analysis.
‡Number of transgenic piglets per total number of piglets in first litter.
§Expression levels detected over two 55-day lactations.

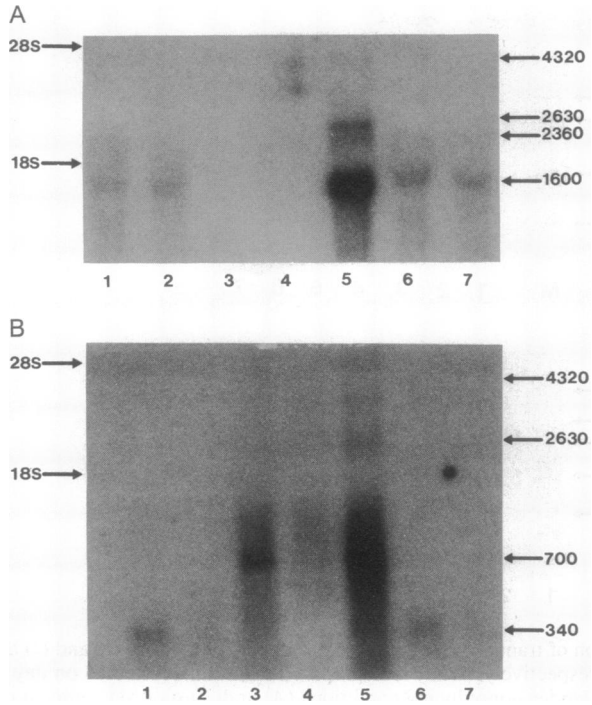

FIG. 3. Northern analyses of pig mammary tissue and human liver RNA. Arrows on the left indicate the positions of 18S and 28S rRNA bands (1867 nt and 5025 nt, respectively); arrows on the right indicate the estimated band sizes in nt. (*A*) Northern blot probed with ^{32}P random primer-labeled hPC cDNA. (*B*) Northern blot probed with ^{32}P random primer-labeled WAP cDNA. Lanes: 1 and 2, 10 μg of human liver total RNA isolated from two different liver samples; 3–5, 10 μg each of control pig, transgenic pig 29-1, and transgenic pig 29-2 mammary gland total RNA, respectively; 6 and 7, replicate samples of lanes 1 and 2.

appears to be about 100- to 1000-fold more intense in the RNA from transgenic pig 29-2 mammary gland than in RNA from human liver.

Northern analysis using ^{32}P random primer-labeled WAP cDNA as the probe (Fig. 3*B*) was done using the same RNA samples as above. Bands corresponding to about 700 nt were present in the mammary RNA of the control pig and transgenic pig 29-2 (lanes 3 and 5, respectively). In addition, two very faint bands of about 2630 and 4320 nt in size were detected in the RNA from pig 29-2 when probed with WAP cDNA.

Protein Analysis. Antigen levels detected by ELISA using polyclonal capture ranged from 200 μg/ml to 1000 μg/ml in two lactations of pig 29-2 and from 140 μg/ml to 650 μg/ml in two lactations of pig 83-1 (Table 1). ELISA using HPC4-Mab immunocapture showed hPC antigen levels ranging from 10 μg/ml to 420 μg/ml and 50 μg/ml to 260 μg/ml in transgenic pig milk from the first lactations of animals 29-2 and 83-1, respectively (Table 1). The antigen level detected by either polyclonal or monoclonal ELISA steadily increased during each lactation for pig 29-2 but was relatively constant for pig 83-1 (data not shown). The milk from four additional transgenic pigs exhibited hPC antigen levels ranging from none detected to 8 μg/ml (by polyclonal capture ELISA). Western blot analyses using WAP polyclonal antibody showed that no WAP antigen was produced in the milk of either control or transgenic pigs (data not shown).

Purification yields for successive immunosorption treatments of starting whey from animal 29-2 (having 1 g of hPC antigen per liter) are given in Table 2. The total HPC4-Mab population capturable by these treatments represented 82% of the total antigen detected by the polyclonal ELISA. The first two immunosorption steps recovered about 63% of the polyclonal rhPC population detected by ELISA, whereas the successive immunosorption at pH 8.5 recovered an additional 19%. About 15% of the polyclonal rhPC antigen was detected in the third immunosorbent fall-through fraction. The sum of residual hPC antigen in whey depleted by the successive HPC4-Mab immunosorptions and the antigen levels from each immunofraction totaled 97% of the antigen detected in the starting whey. Recombinant protein C was also purified from whey taken on different days of the first two lactations for pig 29-2 and pig 83-1.

Coomassie blue-stained SDS/PAGE gels and Western blot analysis (using polyclonal antisera for detection) of whey and immunofractionated samples from milk (animal 29-2) having 1 g of hPC antigen per liter are presented in Fig. 4. The starting whey (lane 2) is compared to rhPC obtained by two successive immunopurifications using HPC4-Mab at pH 7.2 (lanes 3 and 4, respectively) followed by a third immunosorption at pH 8.5 (lane 5) and the remaining rhPC not immunosorbed by the HPC4-Mab (lane 6). Comparison of the scanning densitometries of Coomassie blue-stained SDS/PAGE gels (Fig. 4*A*) with analogous Western analysis (Fig. 4*C*) indicated that all immunocaptured products were greater than about 70% pure (Table 2). Some degradation of whey proteins appeared to be present in the final affinity fall-through fraction as evident by smearing in Coomassie blue-stained SDS/PAGE gels under nonreducing and reducing conditions (Fig. 4 *A* and *B*). Under nonreducing conditions (Fig. 4*C*), the rhPC (lanes 2–6) migrated slightly faster than hPC (lanes 1 and 7). An apparent molecular mass of about 58 kDa was seen for hPC and the molecular mass of the rhPC was ≈55 kDa.

The Western blot analysis using reduced conditions revealed several differences between the rhPC and hPC. (*i*) The rhPC of starting material (Fig. 4*D*, lane 2) and immunoeluates (lanes 3–5) contained more single-chain material than did hPC (lanes 1 and 7). Table 2 presents the single-chain content obtained by diffuse reflectance densitometry of Western analysis from reduced SDS/PAGE. The single-chain content

Table 2. Purification of rhPC from transgenic pig whey containing 1 g of hPC antigen per liter

Step	% hPC antigen recovered*	% purity†	% single-chain content†	Anticoagulant activity,‡ units/mg
Starting whey	100 ± 5	<1	37 ± 5	ND
Immunofraction 1	38 ± 3	>70	30 ± 5	291 ± 5
Immunofraction 2	25 ± 5	>70	32 ± 6	107 ± 12
Immunofraction 3	19 ± 6	>70	24 ± 4	88 ± 15
Fall-through from eluate 3	15 ± 5	<1	3 ± 0.6	ND
hPC reference	—	>90	3 ± 0.1	271 ± 8

ND, not determined due to high background activity.
*Polyclonal antigen ELISA values with standard errors from three dilutions of a single sample.
†Calculated from densitometric comparison of Western analysis and 0.125% Coomassie-stained SDS/PAGE gels under nonreduced conditions for two replicate applications.
‡Standard errors as calculated from delay in APTT for two dilutions of a single purification product.

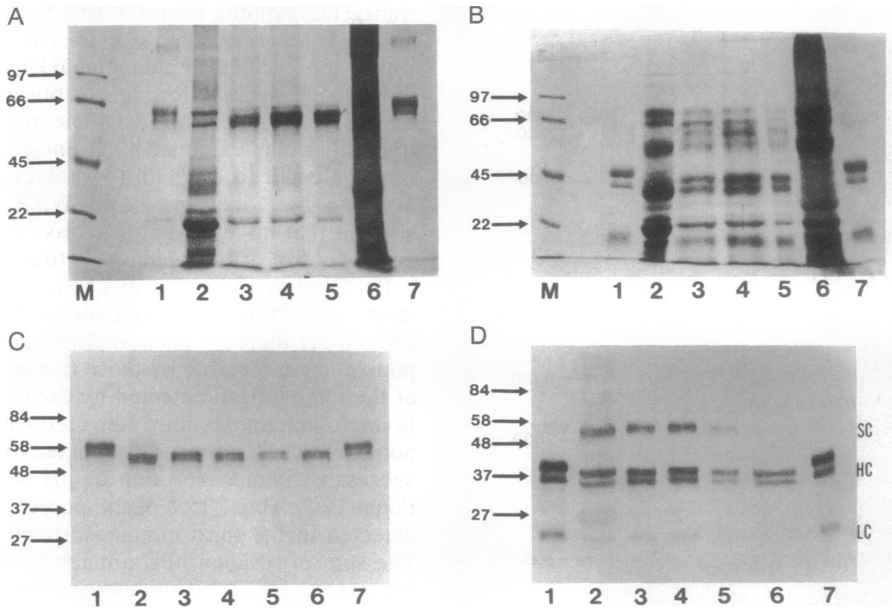

FIG. 4. SDS/PAGE and Western analysis of rhPC from the second lactation of transgenic pig 29-2. Nonreducing conditions (*A* and *C*) and reducing conditions (*B* and *D*) are shown for SDS/PAGE and Western blot, respectively. Whey was prepared from milk collected on day 53 of lactation. Purified samples were applied with 5 μg of hPC antigen per well under nonreducing conditions (*A*) and 7 μg of hPC antigen per well under reducing conditions (*B*); starting whey and final affinity fall-through with ≈1 μg of hPC antigen and 50 μg of total protein is evident for nonreducing (*A*) and reducing (*B*) conditions. Samples analyzed by Western blot were applied with 200 ng of hPC antigen per well under nonreducing conditions (*C*) and 300 ng of hPC antigen per well under reducing conditions (*D*). Arrows indicate positions of molecular mass standards in kDa; M, molecular mass standard mixture. Lanes: 1 and 7, American Red Cross reference hPC immunopurified from normal pooled donor plasma (lot no. 28309018); 2, starting whey (day 53); 3, immunofraction 1 obtained by loading starting whey at pH 7.2 using an estimated 3-fold overload of immunosorbent capacity; 4, immunofraction 2 obtained by loading of first fall-through at pH 7.2 using 10% of immunosorbent capacity; 5, immunofraction 3 obtained by loading of second fall-through at pH 8.5 using 10% of immunosorbent capacity; 6, final affinity fall-through from third loading. SC, single-chain hPC forms; HC, heavy-chain hPC forms; LC, light-chain hPC forms.

was similar in the starting whey (lane 2) and the first two immunosorption eluates (lanes 3 and 4) with about 30–37% single chain. However, the eluate from the HPC4-Mab immunosorption at pH 8.5 (lane 5) and the remaining fall-through fraction (lane 6) contained less single chain—24% and 3%, respectively. hPC contained only 3% single-chain species (lanes 1 and 7). (*ii*) The molecular mass of the putative heavy-chain forms of rhPC (about 35–38 kDa) were about 2–3 kDa lower than that of hPC (about 36–40 kDa). The molecular mass of the light chain of rhPC was about 18–20 kDa and appeared to be about 1–2 kDa lower than that of hPC. The molecular mass of the single chain of rhPC was about 55–58 kDa and appeared to be about 1–3 kDa lower than that of hPC.

Table 2 presents the specific anticoagulant activity based upon delay in APTT due to increasing amounts of normal reference plasma pool (NRPP), purified hPC from NRPP, and rhPC obtained by successive immunosorptions from whey (day 53, second lactation, pig 29-2) containing 1 g of rhPC antigen per liter. The theoretical specific anticoagulant activity of hPC in NRPP is 250 units/mg by definition (7, 16). The specific anticoagulant activity of 271 ± 8 units/mg was determined for hPC immunopurified from NRPP (Fig. 4, lanes 1 and 7). The specific anticoagulant activity of rhPC from the first immunosorption (Fig. 4, lane 3) was determined to be 291 ± 5 units/mg. The specific anticoagulant activity of rhPC from the second immunosorption (Fig. 4, lane 4) was determined to be 107 ± 12 units/mg. The specific anticoagulant activity of rhPC from the immunosorption at pH 8.5 (Fig. 4, lane 5) was determined to be 88 ± 12 units/mg. The specific anticoagulant activity of recombinant protein C (corresponding to immunofraction 1 and having a 30–60% recovery of hPC antigen predicted by polyclonal ELISA) from milk obtained on different days of lactation from the first and second lactations of pigs 29-2 and 83-1 ranged from 70% to 150% of that obtained for hPC reference material (data not shown).

DISCUSSION

Analysis of Southern blots of DNA from pig 29-2 and offspring revealed different banding patterns among the piglet DNA. Upon complete digestion, these bands could be reduced to a single band (data not shown). DNA from the offspring of pig 29-2 had different banding patterns and intensities. The banding pattern of DNA from only one of the piglets resembled that of the parent. Transgene insertion into multiple chromosomes seems to have occurred for founder pig 29-2, resulting in differing copy numbers being inherited by the offspring. Judging from the banding patterns and intensities observed for the piglet DNA, integration in sow 29-2 appears to have occurred on at least three chromosomes, with different copy numbers at each insertion site.

Northern analyses (Fig. 3 *A* and *B*) showed the presence of a major transcript of about 1480 nt was detected (when hybridized with the hPC cDNA probe) in the RNA of pig 29-2. In the human liver RNA sample, a single band of 1600 nt was detected. The relative amounts of hPC mRNA from pig 29-2 mammary gland and human liver hPC mRNA agree with the relative amounts of protein C produced by the two tissues. In pig 29-2 RNA, another band of about 2360 nt was specific for hPC only. Other transcripts of about 2630 and 4320 nt (very faint) were detected in both Northern blots, indicating they may be read-through transcripts containing hPC and WAP sequences.

In the present study, the mouse WAP gene was used to direct the expression of the hPC cDNA into the milk of transgenic swine, at levels that were about 1000-fold greater in transgenic pigs than in transgenic mice containing the same construct (8). This result was notable because in previous

studies high-level production of heterologous proteins in milk was achieved only in transgenic animals carrying either genomic (2, 17) or minigene constructs (3). Our results show that a cDNA may be used to obtain high-level expression of heterologous proteins in the milk of transgenic pigs.

The highest expression levels of rhPC in the milk of transgenic pigs using the cDNA of hPC were similar to the expression levels reported for the WAP using the intact genomic mouse WAP gene in transgenic pigs (2). A constant level of WAP of about 1 g/liter was found in milk of transgenic pigs over a 26-day lactation (17); rhPC (polyclonal population) increased about 2-fold during the first 26 days and >5-fold during the entire 55-day lactation of pig 29-2, whereas the rhPC in the milk of pig 83-1 varied about 2.5-fold. Since WAP and rhPC transgenic pigs contained about 10–20 copies of the transgene, differences in regulation may have been due to the location of integration as well as the differences between the WAP and WAP-hPC gene structures.

The molecular mass of the single, heavy, and light chains of rhPC appeared to be similar to, but slightly lower than, those for hPC. This may be due to differences in carbohydrate content and structure. The rhPC contained a significantly larger population of single-chain material, which may indicate a rate limitation in posttranslational removal of the dipeptide Lys-Arg at positions 156 to 157 (7).

The presence of different rhPC populations was also evident from the differences in antigen content detected by polyclonal and HPC4-Mab ELISAs and the presence of different immunofractions obtained using the HPC4-Mab. The HPC4-Mab binds the activation peptide of hPC at pH 7.3 and thus provides a measure of the presentation of a domain essential for conversion of zymogen hPC to active serine protease form (15). Each of the immunofractions possessed different anticoagulant activities, but activity did not correlate well with single-chain rhPC content. The most active fraction represented about 38% of the hPC antigen and contained 30% single-chain material. This suggests that a significant portion of the single-chain material contained in this fraction may be biologically active or that some heterodimeric forms are hyperactive. The lower activities of the second and third fractions may be a result of nonnative conformations or insufficient γ-carboxylation.

The anticoagulant activity of protein C is dependent upon proper γ-carboxylation of the membrane binding domain that occurs in the light chain (18). To determine whether γ-carboxylation had occurred properly in rhPC, its anticoagulant activity was assayed *in vitro* by APTT. This assay simulates coagulation *in vivo* by initiating clotting in a mixture containing calcium, phospholipid membrane, and the proteins associated with hemostasis (16). As much as 38% (or 380 μg/ml) of the porcine rhPC may be sufficiently γ-carboxylated, as judged by the specific activity of immunofraction 1 by APTT relative to that of hPC. The transgenic pigs studied here had milk letdown about every hour and hence the maximum rhPC secretion rate occurred at about 1000 μg/ml per hr. The amount of active rhPC secreted by the pigs (about 380 μg/ml per hr) is significantly higher than the secretion rates of about 1 μg/ml per hr produced by human kidney 293 cell lines (7). These results provide evidence of gla formation not previously known to occur at high levels in mammary tissue.

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