Seminal vesicle production and secretion of growth hormone into seminal fluid

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Production of foreign proteins in the tissues of transgenic animals represents an efficient and economical method of producing therapeutic and pharmaceutical proteins. In this study, we demonstrate that the mouse P12 gene promoter specific to the male accessory sex gland can be used to generate transgenic mice that express human growth hormone (hGH) in their seminal vesicle epithelium. The hGH is secreted into the ejaculated seminal fluids with the seminal vesicle lumen contents containing concentrations of up to 0.5 mg/ml. As semen is a body fluid that can be collected easily on a continuous basis, the production of transgenic animals expressing pharmaceutical proteins into their seminal fluid could prove to be a viable alternative to use of the mammary gland as a bioreactor.

Keywords: transgenic mice, agriculture, genetic engineering, bioreactor

The advent of transgenic technologies has made the production of pharmaceutical proteins by transgenic animals an attractive alternative to the production of such proteins in microbial fermentors or in cultured mammalian cells[1]. Currently, these technologies have advanced to the stage that milk-derived proteins are routinely being expressed by transgenic livestock[2–4]. The mammary gland is generally considered to be the tissue of choice for the production of pharmaceutical proteins because milk is easily collected in large volumes. However, the production of proteins in milk is limited by the relatively long interval from birth to first lactation encountered with domestic livestock, the discontinuous nature of the lactation cycle, and the substantial time and material investments required to produce transgenic cattle[5], the species from which milk is most easily and efficiently collected.

Therefore, other forms of collectable body fluids that could be used for the production of foreign proteins in transgenic animals are being considered. To date, the possibility of isolating foreign proteins from the blood of transgenic pigs has been explored[6], as has the idea of using the bladder as a bioreactor by engineering urethelium production and secretion of a foreign protein into the urine[7].

The seminal fluid of the male ejaculate represents an alternative body fluid that is commonly and easily collected from domestic livestock. Of particular interest is the seminal fluid of the pig, as a boar ejaculates the largest volume of seminal fluid of all domestic livestock, reaches sexual maturity at 110–125 days of age, and is able to produce semen on a continuous basis[8]. The seminal vesicle, prostate, and bulbourethral glands, which are among the accessory sex glands, are responsible for the majority of liquid and proteins found in semen[9].

One type of protein known to be expressed by the accessory sex glands of several mammalian species[10,11], including the boar[12], is a group of protease inhibitors that are believed to protect the genital tract from proteolytic damage[13] and to play a role in fertilization[14,15]. The mouse form of this protease inhibitor, P12, has been well characterized in the male glands and demonstrates testosterone-dependent activity in the accessory sex glands with a lesser constitutive expression in the pancreas[16,17]. The P12 upstream 5′ regulatory sequence has also been identified, and its activity extensively is well characterized[18,19].

In this study, we explore the possibility of using the male accessory sex glands, particularly the seminal vesicle, as a bioreactor using the mouse P12 gene promoter to drive the production of a foreign protein into the seminal fluid of transgenic mice. Human growth hormone (hGH) was chosen as the protein of expression because detection systems for this protein are readily available and the effects associated with ectopic transgene expression are well characterized[20].

Results

Production of transgenic mice. The microinjection of the P12-hGH DNA construct resulted in the production of four transgenic founder mice (three male and one female). The female founder died shortly after producing her first litter, which contained no transgenic pups. One of the male founders did not produce detectable levels of hGH in its semen and failed to pass the transgene on to its offspring, suggesting that this founder was mosaic. The remaining two transgenic males (founders 1027 and 2097) produced detectable levels of hGH in their seminal fluid and were mated to nontransgenic females to produce the P12-hGH-1027 and P12-hGH-2097 lines. These founders proved to be nonmosaic as evidenced by the facts that: (1) they passed the transgene on to approximately 50% of their offspring, and (2) hGH production by founders and their progeny was similar. Reduced libido in the transgenic founders and their transgenic male progeny, a common problem associated with animals overexpressing GH[20,21], made the production of offspring and physiological evaluation somewhat arduous, particularly in the P12-hGH-2097 line.

Expression of the hGH transgene. Transgene expression in the testes, ovaries, uterus, seminal vesicle, pancreas, spleen, kidney, liver, heart, and brain was assessed by northern blot analysis. Production of hGH mRNA was detectable in the seminal vesicles and kidneys of males from both transgenic lines (Fig. 1), and in the kidneys of transgenic females. A degree of variation in the levels of expression was observed between lines (Fig. 2). The unique band observed in the seminal vesicle and kidney was of similar size to the hGH mRNA standard present in the human pituitary. No pancreatic expression of the transgene was detected, despite reported constitutive expression of the endogenous P12 gene in this tissue[22]. Quantification of

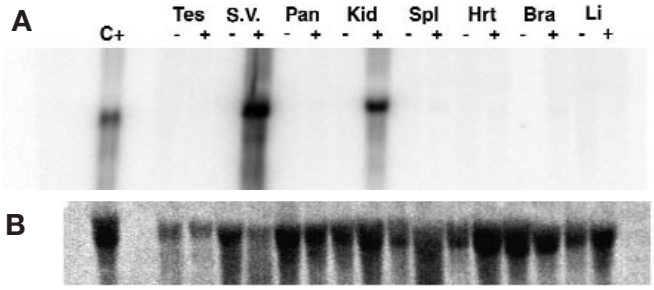

Figure 1. (A) Northern blot analysis of RNA extracted from various tissues of nontransgenic (-) and transgenic (+) male mice from the P12-hGH 2097 line. The probe used consisted of ^{32}P-labeled hGH cDNA. Each lane contains 15 μg of total RNA from the tissue indicated (Tes, testes; S.V., seminal vesicles; Pan, pancreas; Kid, kidney; Spl, spleen; Hrt, heart; Bra, brain; Li, Liver), except the positive control (C+), which contained 1 ng of human pituitary poly A+ RNA with nontransgenic liver total RNA. (B) 18S ribosomal RNA subunit.

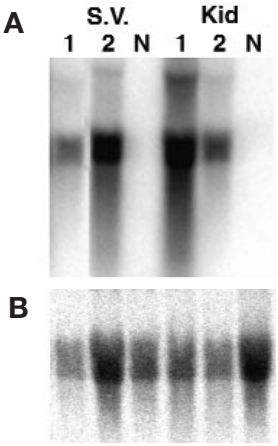

Figure 2. (A) Northern blot analysis of RNA extracted from the seminal vesicles (S.V.) and kidneys (Kid) of male mice from the two P12-hGH transgenic lines (1, line 1027; 2, line 2097) and a nontransgenic male (N). The probe used consisted of a ^{32}P-labeled hGH cDNA. Each lane contains 15 μg of total RNA from the tissue indicated. (B) 18S ribosomal RNA subunit.

the major bands revealed that, in the P12-hGH-2097 line, expression in the seminal vesicle was approximately two orders of magnitude greater than in the kidney. In the P12-hGH-1027 line, the opposite was true: Expression in the kidney was approximately three times that observed in the seminal vesicle.

Immunohistochemical staining revealed that the hGH mRNA expressed in the seminal vesicle and kidney was indeed translated into the hGH protein (Fig. 3). In the seminal vesicle, hGH was immunolocalized in the lumen and on the lumen epithelial folds, where expression of endogenous P12 is normally found[16]. In the kidney, the hGH protein granules were associated with the epithelium of the proximal tubes, which are in direct contact with the blood circulatory system. As P12 promoter activity is generally considered to be specific to the accessory sex glands, such as the seminal vesicle, expression of the hGH transgene in the renal tissues was unexpected.

Secretion of hGH into the seminal fluid. The secretion of hGH into the seminal vesicle lumen contents and in ejaculated seminal fluids was determined by radioimmunoassay (RIA). Because of the lack of an efficient technique for collecting mouse ejaculate, initial measurements were performed on extracts of the vaginal plug found in the female following mating. Evaluation of vaginal plug extracts from the transgenic founder males revealed hGH concentrations for founder 1027 of 1.19 ± 0.45 μg/ml (mean ± s.e.; n = 5; maximum, 2.52 μg/ml) and for founder 2097 of 10.36 ± 4.34 μg/ml (n = 3; maximum, 14.67 μg/ml). The offspring of each founder demonstrated similar levels of hGH expression in their vaginal plug extracts to that of the founders. The male mice were killed and the contents of their seminal vesicles examined, revealing a similar trend but amplified by a factor of greater than 100 (Table 1). A time course study of vaginal plug extract hGH concentrations for the P12-hGH-1027 transgenic line revealed that hGH expression during development was comparable to that of the endogenous P12 protein[16]. Minimal levels of hGH were detectable from four to seven weeks of age, followed by a dramatic increase in hGH secretion up to 12 weeks of age, at which time the hGH levels reached that of mature males (Fig. 4).

The total protein in the vaginal plug extracts and seminal vesicle contents did not differ between the transgenic lines (Table 1). Moreover, it was not different from that in nontransgenic control males, which demonstrated vaginal plug extract protein contents of 613.79 ± 101.25 μg/ml (n = 5) and seminal vesicle concentrations of 86.33 ± 12.71 mg/ml (n = 3). The hGH protein in the liquids tested represented up to 0.93% of the total protein, but did not dramatically alter the amount of protein present.

The circulating concentrations of hGH were substantial, with 12-week-old male mice demonstrating 247.09 ± 87.73 ng/ml (n = 6) and 513.70 ± 87.73 ng/ml (n = 3) for the 1027 and 2097 lines, respectively. The females of these lines demonstrated serum hGH concentrations of 299.40 ± 64.74 ng/ml (n = 5) and 502.78 ± 82.93 ng/ml (n = 7), respectively. At no time was hGH detectable in the serum, vaginal plug extracts, or seminal vesicle contents of the nontransgenic control animals. Although GH overexpression has been found to be negatively correlated with female fertility[23], the P12-hGH transgenic females did not possess adequately elevated circulating levels of hGH to dramatically impair their reproductive function. The growth rates of the P12-hGH transgenic mice was affected by the increase in circulating hGH. The weights of 12-week-old male and female mice from the 1027 and 2097 lines, respectively, were 123% and 177% that of nontransgenic mice of the same age.

Discussion

The data presented illustrate, for the first time, that the 4.0 kb 5′ regulatory region of the mouse P12 gene can be used to direct the expression of a foreign protein into the seminal fluid of a transgenic animal. These results strongly support the proposal that the male accessory sex glands can be used as a bioreactor.

The limited expression of the P12-hGH transgene in tissues other than the seminal vesicle indicates that the 4.0 kb P12 5′ regulatory region contains the primary elements required to achieve transgene expression specific to the accessory sex gland. The circulating levels of hGH in the P12-hGH transgenic mice most likely

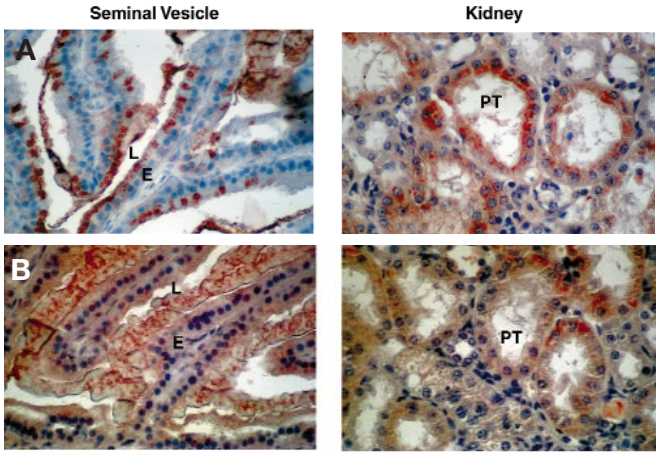

Figure 3. Immunohistochemical staining patterns of hGH on sections of the seminal vesicle and kidney of transgenic male mice. (A) Human growth hormone detected with rabbit antisera to hGH. (B) Specimens were treated as in (A) except hGH antiserum was replaced with normal serum. The hGH was localized on the epithelium (E) and in the lumen (L) cavity of the seminal vesicle and on the epithelium of the kidney proximal tubules (PT).

can be attributed to the ectopic expression of the transgene in the kidney, as this was the only tissue expressing the hGH mRNA in the female. Furthermore, in the male transgenic mice, the level of hGH present in the serum was independent of the hGH in the seminal fluid, as suggested by the fact that hGH serum levels at 4 weeks of age were similar to those at 12 weeks (Fig. 4). Given that serum hGH concentrations do not increase with the onset of the transgene's expression in the seminal vesicle, it is likely that the transgenic protein remains isolated in this gland and does not cross into the circulation. Finally, the use of matrix attachment regions that insulate the gene of interest from the influence of its position in the genome could aid in eliminating ectopic expression, as previous groups have achieved tissue-specific transgene expression in an accessory sex gland in this manner[24].

The hGH concentration in the fluid of the seminal vesicle was found to be as great as 0.5 mg/ml, representing up to 0.93% of the total protein in this pre-ejaculatory seminal fluid. Though a limited indicator of the total hGH present in the ejaculate, the use of vaginal plug extracts did demonstrate that hGH was also present in the mouse ejaculate. As the seminal vesicle is the primary contributor of protein in rodent's semen[9], it can be assumed that the actual concentration of hGH in the ejaculate would be comparable to that found in the seminal vesicle contents. Thus, the rate of hGH production observed in the P12-hGH transgenic mice greatly exceeds the 500 ng/ml of hGH reported in the urine of transgenic mice used as a model for the bladder as a bioreactor[7], but falls slightly short of the 1.0 mg/ml mammary gland production of hGH observed with transgenic mice[25].

We have demonstrated the production of significant hGH protein levels in the seminal fluid of transgenic mice using the P12 gene promoter. However, it should be noted that the complete protein profile of mouse semen is not fully characterized. Thus, there may exist other major seminal fluid proteins, the gene promoters of which could be used to express even greater concentrations of foreign protein in semen.

A domestic boar ejaculates 200–300 ml/ejaculate with a total protein concentration of 30 mg/ml and can ejaculate two to three times per week, year round[9]. Therefore, a single transgenic pig expressing a foreign protein under the control of a similar promoter specific to the accessory sex gland at a rate of 1.0 mg/ml could produce 22.4 g of protein/year. The collection and handling of boar semen is a well-established process, performed on a large scale at swine artificial insemination units worldwide, with more than 1,800 boars maintained by such units in the Canadian province of Québec alone[26]. In addition, the prolificacy and relatively short generation period of the pig means that a herd of transgenic pigs producing a pharmaceutical protein in their semen could be generated in a relatively short period of time. We have calculated that, under ideal conditions, a herd of 60 such pigs could be established in approximately two years. This contrasts greatly with the seven years previously calculated to generate a herd of transgenic cows producing the same protein in their milk[5].

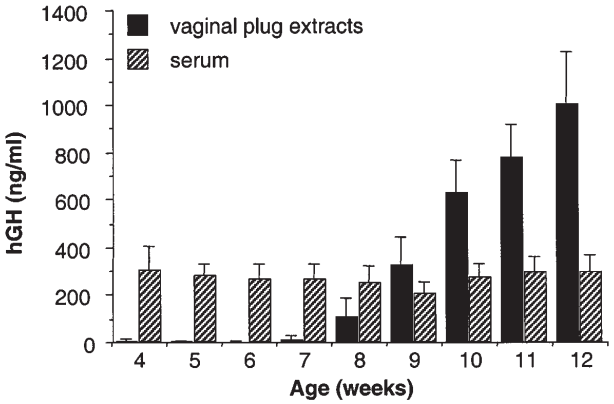

Figure 4. Time course of hGH concentration in the vaginal plug extracts and serum from P12-hGH 1027 male transgenic mice. Each bar of the graph represents the mean ± standard error of at least three mice.

An important consideration in the production of pharmaceuticals in the body fluids of transgenic animals is the processes involved in isolating and purifying the protein of interest. The isolation of recombinant proteins from milk, a complex biological mixture, has proved to be somewhat problematic, as the process is complicated by the presence of casein micelles and fat globules, which prevent filtration and interfere with standard protein separation methods[27]. Semen, though complex, does not pose the same challenges as milk on the level of protein isolation. From a recombinant protein point of view, the primary contaminating factor in semen is spermatozoa, which are easily eliminated by low-speed centrifugation. Furthermore, because of its established role in spermatozoa survival and function, seminal fluid has been well studied. As a result, the isolation of endogenous proteins from boar seminal fluid for characterization purposes is well documented[12,28–31]. As with milk[27], once the solid contaminants have been removed from seminal fluid, protein extraction can be achieved using established separation techniques. The extraction approach to isolating and purifying a recombinant protein from the semen of a transgenic boar will depend greatly on the character of that protein. However, the fact that the endogenous protein profile of porcine seminal fluid is well characterized will aid in planning recombinant protein purification techniques and creating specially designed fusion proteins to greatly simplify these processes[32].

To date, the mammary gland[4], blood[6], bladder[7], and now the male accessory sex glands have all been considered as bioreactors for pharmaceutical proteins. Each of these systems has unique advantages and disadvantages. However, an aspect of mass protein production in the tissues of transgenic animals, yet to be fully explored, is these tissues' ability to execute complex posttranslational modifications. This process varies considerably from one protein to another and may also differ dramatically from tissue to tissue. Therefore, this potential limitation may necessitate the

Table 1. Human growth hormone (hGH), total protein, and hGH as a percentage of total protein in the vaginal plug extracts and seminal vesicle lumen contents of P12-GH transgenic offspring[a]

	Vaginal plug extracts				Seminal vesicle contents			
Line	n[a]	hGH (μg/ml)	Protein (μg/ml)	hGH as % of protein	n[b]	hGH (μg/ml)	Protein (mg/ml)	hGH as % of protein
1027	5	1.83 ± 0.76	560.77 ± 84.84	0.30 ± 0.13	3	241.06 ± 105.28	87.38 ± 12.01	0.24 ± 0.09
2097	5	3.09 ± 1.03	667.46 ± 177.82	0.47 ± 0.22	3	472.11 ± 55.35	66.59 ± 12.02	0.72 ± 0.12

[a]hGH, protein, and hGH as percentage of protein values are given as mean ± standard error.
[b]n is the number of mature (>80 days) offspring sampled. Each individual animal was sampled only once.

development of several unique transgenic bioreactor systems, each suited to the production of a certain protein type, based on its post-translational modification needs.

Experimental protocol

Construction of the P12-hGH transgene. The 4.0 kb 5′ regulatory region of the murine P12 gene was isolated from the previously described P12.4000 plasmid[18] and ligated upstream to the hGH cDNA and SV40 small t intron and poly A in the poly-cloning site of the pPolyIII plasmid. The resulting pP12-hGH plasmid was digested with *Not*I to isolate the 5.0 kb transgene for pronuclear microinjection.

Generation of transgenic mice. The collection of mouse embryos (strain B6C/3F1), pronuclear microinjection, and transfer of embryos into pseudopregnant recipients (strain CD-1) were performed using established methods[33]. Offspring were screened for the P12-GH transgene by Southern blot analysis.

Southern and northern blot analysis. Identification of transgenic mice was performed by Southern blot analysis using 20 µg of tail genomic DNA digested with *Hin*dIII. A positive control consisting of nontransgenic *Hin*dIII digested genomic DNA, spiked with 50 pg of the P12-hGH construct, was incorporated into each analysis. Analysis of transgene expression in various tissues was performed by northern blot using 15 µg samples of total RNA. For each analysis, we included a positive control consisting of 15 µg of non-transgenic kidney RNA, spiked with 1 ng of human pituitary poly $(A)^+$ RNA (Clontech, Palo Alto, CA). Northern and Southern blots were probed with a ^{32}P-labeled hGH cDNA insert, and the resulting membranes were evaluated using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). Equal lane loading was determined for northern blots by stripping and reprobing membranes with a ^{32}P-labeled probe specific for the 18S ribosomal subunit.

hGH RIA. The concentration of hGH in vaginal plug extracts, seminal vesicle contents, and serum was determined by RIA. Vaginal plug extracts were produced by placing transgenic and nontransgenic males with Pregnatn Mares' serum Gonadotrophin (PMSG)-hCG synchronized females overnight and collecting the vaginal plug the next morning. The vaginal plug was placed in 0.5 ml of buffer (Tris 10 mM, EDTA 1 mM) for 1 h so that the hGH within the plug could diffuse into solution. Seminal vesicle contents were collected from the seminal vesicles of mature transgenic and nontransgenic male mice with the coagulating gland carefully dissected. Blood samples were collected from mice under anesthesia and centrifuged, and the serum was collected. Samples were kept at -80°C until the time of assay. Concentrations of hGH were determined using a RIA kit specific for the human protein (Immunocorp, Montréal, Québec, Canada).

Immunohistochemistry. Tissues were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sectioned at 6 µm, and stained using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Human growth hormone was detected using rabbit antisera to hGH (NIDDK-anti-hGH-IC-3).

Total seminal fluid protein determination. Total protein in the seminal vesicle contents and vaginal plug extracts was determined using a protein assay kit (Pierce Scientific, Rockford, IL). Bovine serum albumin was used as a standard.

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