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The Isolation and Characterization of the Soluble and Membrane-Bound Porcine Cytochrome b_5 cDNAs

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In some male pigs, there is an increased production of the testicular 16 androstene steroids which end up being concentrated in fatty tissue. When the meat is cooked, a disagreeable odor/flavor is produced, a phenomenon known as "boar taint." All boars selected for food production are castrated even though only ca 10% of boars may be "tainted." This has a high economic cost because castrated pigs convert food into meat less efficiently, the meat is fattier, and there is an increased mortality due to the castration procedure. Recent data has implicated an increased level of cytochrome b_5 in the testes with the increased synthesis of the 16-androstene steroids. As an initial step in analyzing this process, we used 5′ and 3′ RACE PCR procedures to isolate, clone and sequence the cDNAs for the membrane-bound and soluble forms of porcine cytochrome b_5.

Cytochrome b_5 is a small amphipathic protein that plays a role in a wide variety of biological processes including fatty acid elongation (1), oleic acid desaturation (2), P450-linked drug oxidation (3), CMP-N-acetylneuraminic acid hydroxylation (4), steroid biosynthesis (5,6,7), and methemoglobin reduction (8). Cytochrome b_5 has been found in all tissues examined (9), as either a membrane-bound 133aa protein or a soluble 97aa protein, both of which are derived from one mRNA via alternative splicing (9,10).

The amino acid sequence of cytochrome b_5 is highly conserved (11), with an especially high percentage of invariant amino acids (11). There is, however, some divergence of sequence between mammals and birds (ca. 78%), and with plants (ca. 30%)(12). The cDNA sequences of cytochrome b_5 mRNA's from mammals (13,14,15,16), chicken (17), yeast (18) and plants (12, 19) have been elucidated as well as cytochrome b_5 gene organizations for human, rabbit, bovine and chicken (20,21,22,23).

Recently, evidence has implicated increased levels of a small molecular weight form of cytochrome b_5 with the increased production of 16-androstene steroids by pig testes (24). High levels of testicular 16-androstene steroid production is a principle cause of "boar taint," a problem due to the concentration of these steroids in fatty tissues. When the pork is cooked, these steroids cause a disagreeable flavor and odor, e.g., the meat is tainted. The small molecular weight cytochrome b_5 could either be the soluble form of cytochrome b_5, the proteolytic product of the membrane-bound form, or a new "mutant" form. Once this cytochrome b_5 has been identified, it will be possible to study why this small molecular weight form is increased in tainted boars. To begin analyzing this problem, we isolated and characterized the cDNAs for the two forms of pig cytochrome b_5.

MATERIALS AND METHODS

Materials. Pig blood was obtained from a local slaughterhouse and was immediately combined with 1/6 vol. Guanidinium thiocyanate to lyse the blood and prevent reticulocyte maturation. Testicular tissue was obtained from Yorkshire cross Landrace boars (95-115kg) from the University of Guelph Swine Research Center. The animals were slaughtered at the University of Guelph Department of Animal and Poultry Science Abbatoir and the tissues snap frozen in liquid nitrogen and stored at −70°C until use.

Oligonucleotides. The sequences of the primers and the corresponding amino acids (aa) of the liver or reticulocyte cytochrome b_5s are as follows: primer A, AT(A/G/T)CA(A/G)AA(A/G)CA(C/T)AA(C/T)AA(C/T)A, aa 16-22; primer B, ACCCA(A/G)TT(A/C/G/T)GTCCACCA (aa 113-108); primer C, TTCCCCACCAGGATGCTCCTC (aa 48-42); primer D, GGAGTGTCCAACATCCTCAA (aa 69-63); primer E, GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTT(A/C/G) (polyA tail); primer F, CTGCACCACAAAGTGTACGA (aa 29-35); primer G, TCGGAAACTTAAAGGC(A/C/G/T)(A/C/G/T)TGT (aa 97-3′nt of putative reticulocyte exon); primer H, CTAGCACGCCAATGGAC (3′nt region); primer I, GTAGAAGTGATACATCAGGGA (aa 129-123), primer J, GATGTTCCATCACTACATCA (3′nt region); and primer K, GCCCAGAAATAATAGCT (3′nt region).

RT/PCR. Total RNA was extracted by the method of Chomczynski and Sacchi (26). Based on the pig amino acid sequence (11), degenerate primers A and B were designed and used to reverse transcribe and amplify total RNA using the Access RT/PCR kit (Boehringer Mannheim, Indianapolis, IN). Per manufacturer's instruction, 100μg total RNA was combined with 1mM $MgSO_4$, 1μM of each

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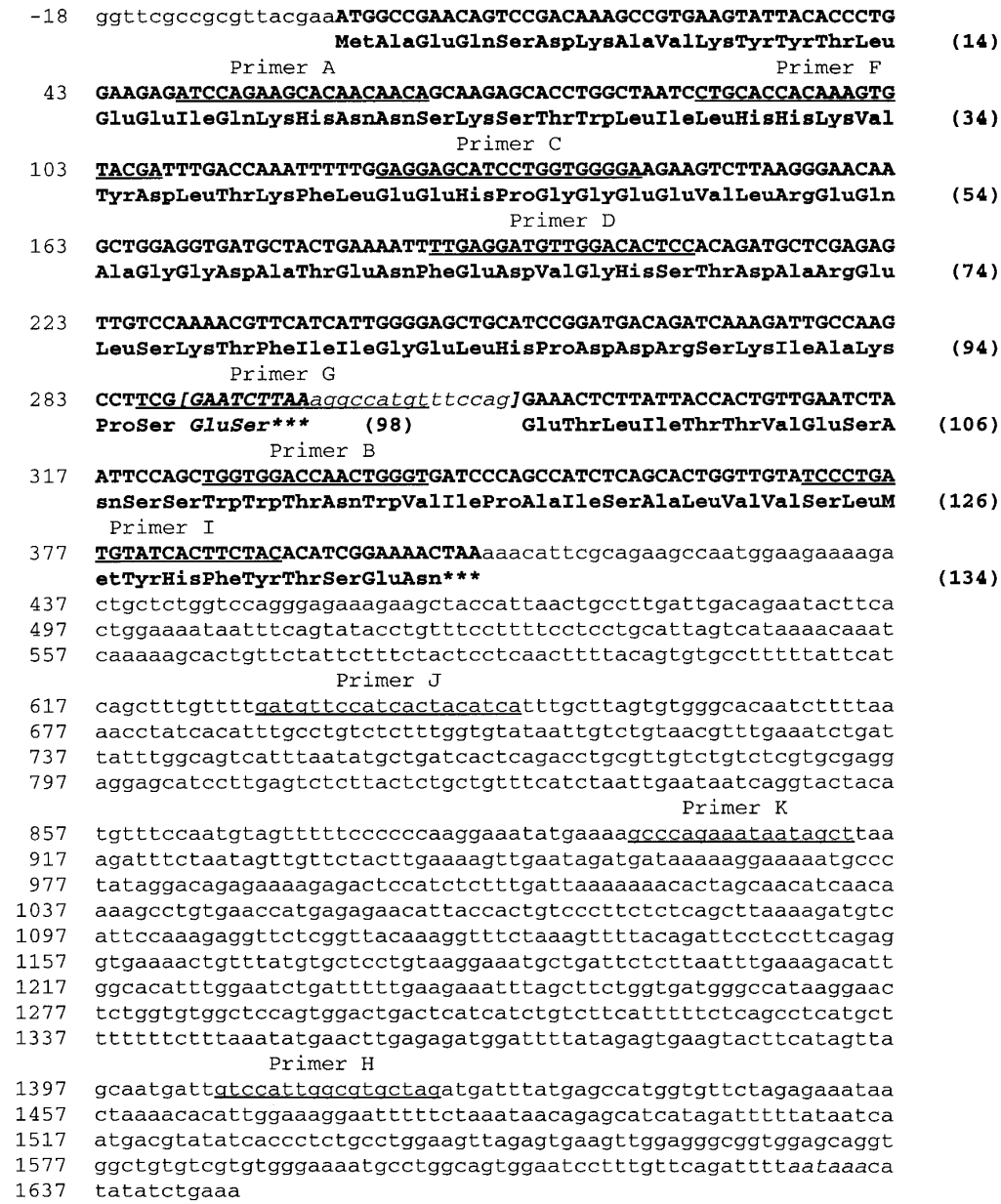

FIG. 1. Nucleotide sequence and deduced amino acid sequences for the pig membrane-bound cyt b_5 cDNA from testis and the soluble cyt b_5 cDNA from blood. The regions used for the design of the oligonucleotide primers are underlined.

primer, 0.2mM dNTP mix, 1X AMV/Tfl reaction buffer, and 0.1u of AMV reverse transcriptase and Tfl DNA Polymerase in a 50μl reaction. The PCR consisted of 35 cycles of denaturing for 2 min. at 94°C, annealing for 2.5 min. at 48°C, and extending for 1.5 min. at 72°C, with a final 5 min. extension step at 72°C. 8μl of the PCR products were analyzed by electrophoresis on a 1% agarose gel.

5′/3′ Rapid Amplification of cDNA ends (RACE). Using primers C-F, 5′ and 3′ RACE was carried out using a commercial kit (Boehringer Mannheim). Briefly, the 5′ RACE was performed by synthesizing the first strand cDNA with primer C, tailing that product via terminal transferase and dATP, and then amplifying the first strand with primers D and E. The 3′ RACE was performed in the same fashion using primers E and F. The PCR was carried out in 20 cycles of denaturation for 1.5 min. at 94°C, annealing for 2 min. at 57°C, and extending for 1.5 minutes at 72°C. An aliquot of the PCR products were electrophoresed on a 1% agarose gel and analyzed.

Subcloning. All PCR products were purified using GeneClean (Bio 101, CA) then blunt-ended and phosphorylated (27). After another purification with GeneClean, the Fast-Link DNA Ligation and Screening kit (EpiCentre Technologies, Madison, WI) was used to ligate the PCR product to a Sma cut vector and this product then transformed into competent DH5α cells. DNA was isolated and sequenced using standard procedures (28).

Isolation of full-length cytochrome b_5 cDNA subclones. To obtain full-length cytochrome b_5 cDNA subclones, primers F and I were designed based on the sequence obtained from the 5′ and 3′ RACE subclones and used to RT/PCR total RNA as described previously.

The PCR conditions were the same as in the RT/PCR, except a 58°C annealing temperature was used. The PCR products were analyzed on 1.2% agarose gels, purified, modified and subcloned as described.

Southern blot analysis. The full-length RT/PCR products were transferred from an agarose gel to a nitrocellulose membrane using the descending alkaline transfer protocol (29). After rinsing, the membrane was prehybridized for 45 min. at 55°C then hybridized to an end-labeled oligonucleotide probe (primer C) for 2 hours at 55°C. The probe was labeled in a 20μl reaction containing 1.6μl [α-^{32}P]-dATP (10mCi/ml), 2 pmoles oligonucleotide, 10 units of terminal transferase and 4μl 5× reaction buffer. After hybridization, the membrane was washed and used to expose X-ray film overnight.

Identification of soluble cytochrome b_5 subclones. A degenerate reticulocyte-specific primer G was designed and, after end-labeling, used to screen colonies containing the full-length cytochrome b_5 cDNA derived from the RT/PCR of total blood RNA. The colony hybridization followed standard protocols (28).

RESULTS AND DISCUSSION

To begin characterizing the porcine soluble and membrane-bound forms of cytochrome b_5, the pig cytochrome b_5 amino acid sequence (11) was used to design degenerate primers to regions highly conserved throughout the different mammalian species. Using primers A and B, total RNA isolated from two pig testes samples was reverse transcribed and amplified. The PCR product was subcloned and sequenced. This 279bp clone corresponded to amino acids 16-109 of the pig membrane-bound cytochrome b_5. In order to obtain the remaining sequence, 5′ and 3′ RACE was carried out using specific primers based on the newly obtained sequence. DNA sequencing of the 5′ RACE subcloned products provided 77bp upstream of the ATG site to 130bp into the coding region. The longest 3′ RACE subclone contained an insert ca. 1.7kb in size, which corresponded to the entire 3′-nontranslated tail of the membrane-bound cDNA. The authenticity of this large fragment was verified by the PCR amplification of genomic DNA and the 1.7 kb 3′ RACE subclone using primers F and H within the 3′-nontranslated region. The products were identical in size (data not shown). Our data shows that the pig cytochrome b_5 mRNA is ca 1.75bp in length and has only one AAUAA sequence (Figure 1). This is considerably larger than that of the other mammalian cytochrome b_5 mRNAs. Based on the complete pig membrane-bound cytochrome b_5 sequence, primers F and I were designed to RT/PCR the full-length pig membrane-bound cDNA from total RNA from two testes and blood samples (Figure 2A). The blood PCR product was slightly larger in size than the testis products, consistent with blood containing the soluble cytochrome b_5 mRNA, which has an extra 24bp insert. The identities of the two products were confirmed by Southern blotting and hybridization using a probe specific for the complete cytochrome b_5 sequence (Figure 2B). To specifically identify clones with the soluble cytochrome b_5 cDNA insert, we designed a degenerate primer G based on the following: 1) the relation of the reticulocyte-specific sequence, corresponding to aa 97, 98, Stop, and 3′-nontranslated sequence (9), to the membrane-bound form's sequence at aa 97 and 98; and 2) the conservation of the reticulocyte-specific sequence between human, rabbit and bovine (9,10,21). DNA sequencing of the hybridizing clones provided the pig reticulocyte-specific sequence (Figure 1). The size and sequence of the pig reticulocyte-specific sequence, ie. 24bp, has significant homology with other described reticulocyte-specific sequences (9,10,21). It is interesting to note the recent report of an extra 58bp sequence for the rat soluble cytochrome b_5 mRNA (30); however, no control studies were run, e.g. blood RNA RT/PCR, and it is probable that the 58bp sequence is the result of a library construction artifact.

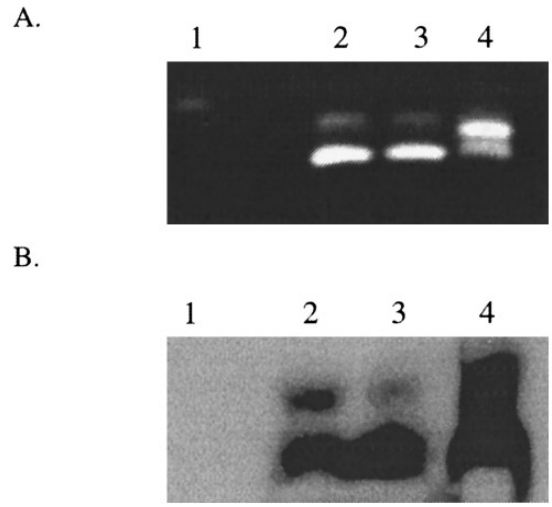

FIG. 2. Agarose gel electrophoresis (A) and Southern blot analysis (B) of pig cytochrome b_5 RT/PCR products using primers F and I. Lane assignments are as follows: 1, 330bp marker; 2, testis A; 3, testis B; 4, blood. In (A), all three samples contain a 300bp product, corresponding to the membrane-bound cytochrome b_5, containing sequence present in both forms of cytochrome b_5, verified the RT/PCR products to be cytochrome b_5.

Future studies will be needed to determine if both forms of cytochrome b_5 are present in the pig testes, the identity of the small molecular weight cytochrome b_5, and how these levels alter in tainted pigs.

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