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Characterization and phylogenetic significance of rhinoceros luteinizing hormone beta (LHβ) subunit messenger RNA structure, complementary DNA sequence and gene copy number

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Abstract

The *luteinizing hormone (LH)* β subunit gene is expressed in the pituitary glands of all mammals, whereas the closely related *chorionic gonadotropin (CG)* β subunit genes have been identified only in primates and equids, and are expressed in placenta. In the case of horses, there is a single-copy *equine (e) luteinizing hormone/chorionic gonadotropin hormone* β subunit gene (*eLH/CGβ*) that (1) is expressed in both pituitary gland and placenta, (2) encodes a characteristic carboxyl terminal peptide (CTP) extension, and (3) transcribes an atypically elongated 5′-untranslated region (*UTR*) in both pituitary and placenta. However, it is not known whether similar expression patterns and gene locus characteristics may be exhibited by other members of the order Perissodactyla (equid, rhinoceros and tapir species). To begin to investigate these possibilities, we undertook analysis of the rhinoceros (rn or rhino) *LH/(CG?)β* gene locus and the *rnLHβ* cDNA. Total RNA isolated from the pituitary gland of a female white rhino was used as template for amplifying *rnLHβ* cDNA by reverse transcription-polymerase chain reaction. Following cloning of the amplified cDNA, nucleotide (nt) and deduced amino acid sequences were determined. The first in-frame stop codon occurred at codon position +122, suggesting that the rnLHβ subunit does not contain a CTP. To assess gene copy number, Southern blot analysis of Indian rhino genomic DNA was performed. The resulting simple hybridization pattern indicated that, as in the horse and donkey, there is a single-copy gene at the *rnLH/(CG?)β* gene locus. Primer extension mapping of the pituitary transcriptional start site of the *rnLHβ* subunit gene revealed an 8 nt 5′-*UTR* which is similar to that reported for the majority of mammalian LHβ transcripts. Northern analysis was consistent with the transcriptional start site findings. We postulate from these data that rhinos diverged from equids prior to the occurrence of the mutations causing CTP expression and adoption of a non-consensus 5′-*UTR*/proximal promoter region. However, these findings do not rule out the possibility of expression of a placental CGβ subunit lacking a CTP in rhinos. © 1997 Elsevier Science B.V.

Keywords: Gonadotropin; Phylogeny; Perissodactyl; RT-PCR cloning; Pituitary; Nucleotide sequence

1. Introduction

Gonadotropins are heterodimeric, glycoprotein hormones that play critical roles in the regulation of vertebrate gonadal function. While production of gonadotropins in most mammals is limited to the pituitary gland, which synthesizes luteinizing hormone (LH) and follicle-stimulating hormone (FSH), a third

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Abbreviations: A, adenosine; α, alpha; aa, amino acid(s); β, beta; b, bovine; bp, base pair(s); C, cytidine; cDNA, DNA complementary to RNA; CG, chorionic gonadotropin; CGβ subunit, luteotropin β subunit peptide produced by placenta; CGβ mRNA, placental transcript encoding CGβ subunit; *CGβ*, gene (DNA) encoding CGβ subunit; CTP, carboxyl terminal peptide; d, dog; dk, donkey; e, equine; FSH, follicle-stimulating hormone; G, guanosine; h, human; kb, kilobase(s); LH, luteinizing hormone; LHβ subunit, luteotropin β subunit produced by pituitary gland; LHβ mRNA, pituitary transcript encoding LHβ subunit; *LHβ*, gene (DNA) encoding LHβ subunit; LH/CGβ subunit, peptide produced in both pituitary and placenta; *LH/CGβ*, gene (DNA) encoding LHβ and CGβ subunits; *LH/(CG?)β*, gene (DNA) encoding LHβ subunit and, potentially, CGβ subunit; nt, nucleotide(s); p, porcine; r, rat; rn or rhino, rhinoceros; RT-PCR, reverse transcription-polymerase chain reaction; S, sedimentation constant; t, turkey; T, thymidine; *UTR*, untranslated region(s).

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gonadotropin, chorionic gonadotropin (CG), is produced in the placenta of equids and primates (Pierce and Parsons, 1981). Gonadotropins are composed of a common α subunit non-covalently bound to a hormone-specific β subunit. Luteinizing hormone and CG are both subclassified as luteotropins because they bind the same gonadal LH/CG receptors and induce similar luteotropic responses.

Most haploid mammalian genomes contain a single gene at the luteinizing hormone (LH) β subunit locus that is expressed only in the pituitary gland (Bousfield et al., 1994) (see Fig. 1). By contrast, this locus in equids and primates is more complex in that it contains one or more luteotropin β subunit gene(s) that have also acquired the capacity for placenta-specific expression. In equids, there is a single *LH/CGβ* gene that is expressed in both the pituitary and placenta (Sherman et al., 1992), whereas in primates there is a single pituitary-specific *LHβ* gene linked to a cluster of *CGβ* genes which are either inactive (pseudogenes) or expressed in a placenta-specific manner (Jameson and Lindell, 1988). In primates, *CGβ* genes have evolved by duplication and mutation of the ancestral *LHβ* gene, followed by repeated replication of the new *CGβ* gene. This has led in humans to the generation of a cluster comprised of one *LHβ* gene linked to six *CGβ* genes (Policastro et al., 1983; Talmadge et al., 1984; Jameson and Lindell, 1988).

Although the ancestral equid β subunit gene did not replicate in the course of evolving a placentally expressed luteotropin, there are other distinctive characteristics shared by *hCGβ* and equid *LH/CGβ* mRNA transcripts (see Fig. 1). One of these unique features is coding of a carboxyl terminal peptide (CTP) extension that results from a frameshift deletion leading to readthrough of the consensus LHβ translational stop codon (codon +122) to a more distal stop codon (Sherman et al., 1992; Sugino et al., 1987). In humans and horses, the frameshift deletion mutation occurred in the area of codon +112 to +114, and led to CTP extensions of 25 and 28 amino acids (aa), respectively. While the physiological importance of this C-terminal domain is not well defined, its presence in both of the lineages known to express CG placentally suggests that a CTP confers a selective advantage.

Another feature shared by equid and primate luteotropin β genes expressed in the placenta is atypical positioning of the transcriptional start site (see Fig. 1). While the 5′-*UTR* regions of most *LHβ* genes are relatively short (6–11 bp), the length of the 5′-*UTR* is 350 for *hCGβ* genes (Talmadge et al., 1984) and 45–62 for the *eLH/CGβ* gene (Sherman et al., 1992). The mechanism of expression of these placental genes is also noteworthy in that transcription of *hCGβ* ignores the consensus TATA and instead uses a TATA-less upstream promoter (Talmadge et al., 1984), while placental eCGβ mRNA synthesis is driven by tandem TATA elements. Thus, there is a suggestive correlation between adoption of non-consensus proximal promoter/5′-*UTR* sequences at human and horse luteotropin β subunit gene loci, and acquisition of the ability to express in placenta.

Chorionic gonadotropin expression is widespread throughout the order Primates (Mwenda et al., 1990; Seshagiri et al., 1994; Crawford et al., 1986; Summers et al., 1993; Steinetz et al., 1992; Hearn et al., 1991) and the family Equidae (Aggarwal et al., 1980; Murphy and Martinuk, 1991; McFarlane et al., 1991). However, it is not known whether CG expression occurs in members of the order Perissodactyla (odd-toed ungulates) other than equids. In addition to the family Equidae, the order Perissodactyla includes the extant families Tapiridae (tapir species) and Rhinocerotidae (rhinoceros species).

Direct experimentation to establish the existence of CG in the rhino by isolating RNA or proteins from early gestational placenta is impractical owing to impediments related to the endangered status and considerable value of remaining captive and wild rhinos. However, other rhinoceros tissues were obtainable, thereby allowing us to begin gathering genetic evidence for or against the possible existence of placental CG in rhinoceros species. By characterizing the rhinoceros *LH/(CG?)β* (the "?" is included in the term rnLH/(CG?)β because it is presently not known whether rhinos produce placental CG in addition to pituitary LH) gene locus and rnLHβ cDNA sequence, and comparing their structures to the homologous primate and equid loci, we anticipated that conclusions regarding evolutionary relationships could be made. Another aim of this work was to characterize a novel luteotropin β subunit locus which, through comparative molecular analysis, might shed light on the poorly understood mechanisms of pituitary-, placenta- and dual pituitary/placenta-specific expression of mammalian *LHβ*, *CGβ* and *LH/CGβ* subunit genes. Specific hypotheses tested in the present study (illustrated in Fig. 1) were that, as in the horse, (1) the rhino luteotropin β subunit locus contains a single-copy *LH/(CG?)β* gene, (2) the *rnLH/(CG?)β* gene(s) encodes a CTP domain, and (3) the 5′-*UTR* of rnLHβ pituitary transcripts is elongated compared to the consensus mammalian LHβ transcript.

2. Results and discussion

2.1. Cloning and analysis of rnLHβ cDNA

White *rnLHβ* cDNA was isolated from pituitary tissue by RT-PCR according to the amplification strategy shown in Fig. 2. The DNA sequence of a 514 bp product and its deduced aa sequence is shown in Fig. 3. To protect against the possibility that the isolated fragment was an unintended amplification product, the sequence

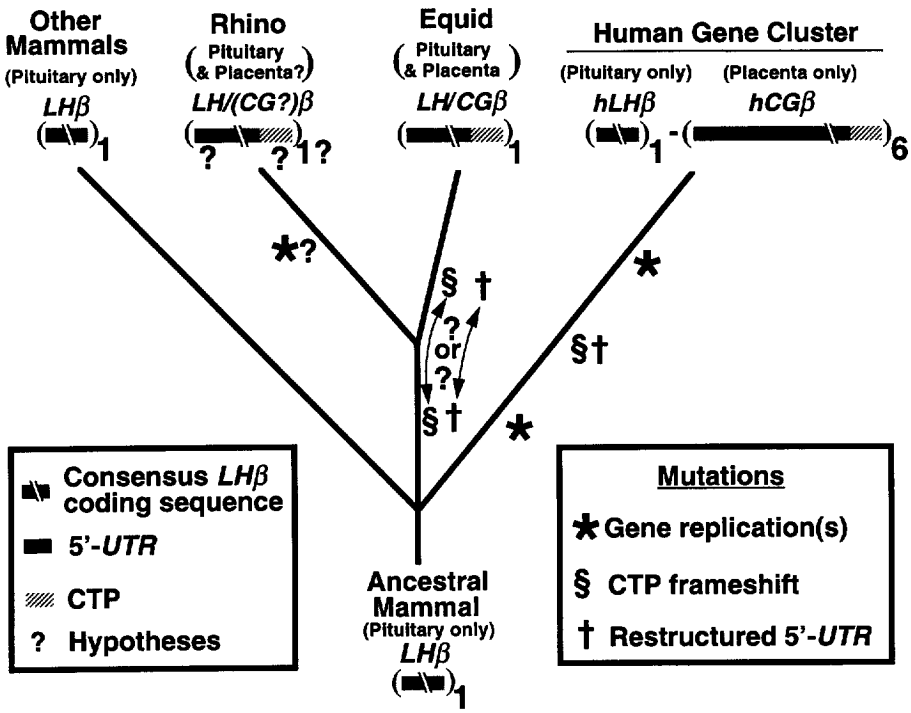

Fig. 1. Representation of mammalian phylogenetic relationships based on luteotropin β gene/mRNA structure. Illustrated are hypotheses relating to gene copy number, CTP expression and length of 5'-*UTR* in rhinos which test the temporal order of specific mutational events relative to the point of divergence of the families Rhinocerotidae and Equidae.

of this DNA was aligned with a panel of nine potentially contaminating vertebrate species' *LHβ*, *CGβ* and *LH/CGβ* coding sequences. The distance matrix shown in Table 1 demonstrates that our proposed sequence is unique, and exhibits percentage sequence similarities that are generally consistent with the expected phylogenetic relationships among the species included in the analysis; the white rhino sequence shows greater similarity to homologous ungulate sequences and less similarity to primate, rodent and avian sequences. Further supporting the contention that the isolated sequence represents bona fide *rnLHβ* cDNA is the fact that no vertebrate pituitary or placental RNA had previously been isolated using either the reagents or laboratory facilities employed in these studies. Thus, the finding of LHβ-like cDNA sequence bearing a polyadenylated tail within the first (partial) amplification product (see Fig. 2, fragment A) strongly suggests that the derived nt sequence reported in Fig. 3A (fragment B) represents *rnLHβ* cDNA.

2.2. *Determination of* rnLH/(CG?)β *gene copy number*

While it was not considered likely a priori that there is more than a single *rnLH/(CG?)β* gene, there is precedent in the primate lineage for gene replication at this locus (Policastro et al., 1983; Talmadge et al., 1984). To determine whether one gene or multiple genes are present in rhinos, Southern blot analysis of Indian rhino genomic DNA was performed (see Fig. 4). The simple banding pattern observed following hybridization of restriction endonuclease-digested genomic DNA with white *rnLHβ* cDNA probe strongly suggests the presence of a single gene per haploid genome. Accordingly, the hypothesis that rhinos carry a single copy gene at the *rnLH/(CG?)β* gene locus was accepted. This finding is consistent with the single-gene condition present in most mammals, including equids, and suggests that the isolated cDNA represents the only white *rnLH/(CG?)β* coding sequence. However, these data do not rule out the possibility that a recent gene replication event occurred in white rhinos, subsequent to this species' divergence from the Indian rhino.

2.3. *Evaluation of peptide structure*

The aa sequence deduced from cDNA sequence reveals an in-frame translational stop site at codon position +122 (see Fig. 3B). The same translational stop site was identified in both of the independently amplified cDNAs, strongly suggesting the veracity of both the reading frame and the positioning of the proposed stop site. This indicates that the white rnLH/(CG?)β subunit mature peptide length (121 aa) matches that of the relatively highly conserved consensus mammalian LHβ subunit. These data, together with confirmation of a single-copy luteotropin β gene, lead to the rejection of the hypothesis that rn LH/(CG?)β

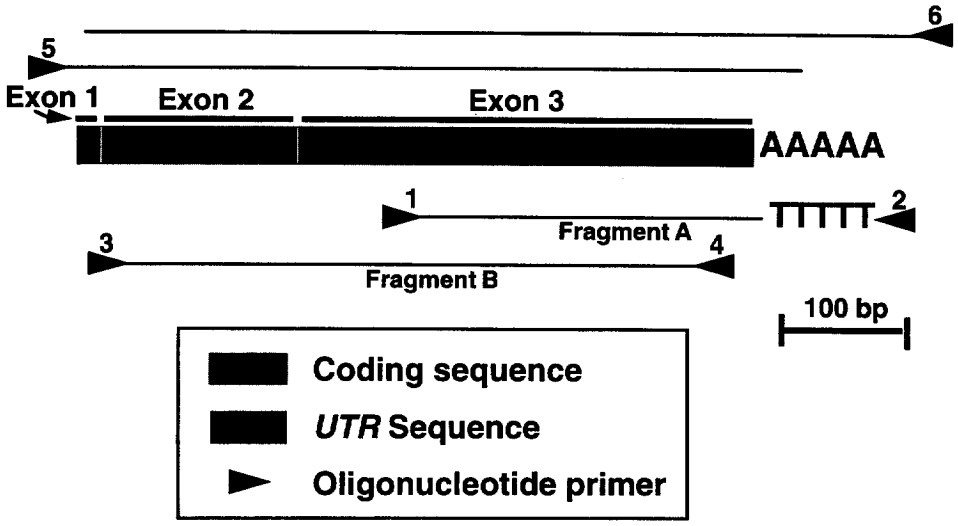

Fig. 2. Amplification and sequence analysis of *LHβ* cDNA from a 31-year-old, female, southern white rhino (*Ceratotherium simum simum*; white Rn-1 in subsequent figures). Triangles represent primers used for PCR or sequencing. Lines extending from triangles depict sequence obtained using a given primer (above mRNA diagram) or PCR-amplified fragments generated from pairs of primers (below mRNA diagram). A procedure for rapid amplification of cDNA 3′-ends (3′-RACE; Gibco) was used according to the manufacturer's specifications. Following reverse transcription of total pituitary RNA using Adapter Primer, primer 1 (an equine-based oligonucleotide; 5′-GTGCGGGTGATGCCTGCCGCCCTGCCGGC) was used in combination with primer 2 (Universal Amplification Primer) to amplify partial cDNA fragment A. Contiguous cDNA fragment B, encompassing the entire mature peptide, was then PCR amplified using porcine-based primer 3 (5′-CACCAAGGATGGAGATGCTCCAGG) paired with homologous primer 4 (5′-TATAAGGAGGGAAGGGAGGGGA) located within the 3′-*UTR* (based on fragment A sequence). The PCR was carried out for 33 cycles (denaturation at 95°C for 1 min; annealing at 62°C for 1 min; extension at 72°C for 2 min). The reaction was terminated with a final extension at 72°C for 20 min. A control RT-PCR reaction lacking reverse transcriptase was also performed on rhino pituitary RNA. Subsequent use of this negative control reaction mix as a source of PCR target resulted in no product formation. After cloning into pCR-Script (Stratagene), fragment B was sequenced in both directions with vector-based primer 5 (5′-GTAAAACGACGGCCAGT; M13/Forward) and primer 6 (5′-AGCGGATAACAATTTCACACAGGA; M13/Reverse) using the Applied Biosystems 373A Automated DNA Sequencer. A similar strategy was subsequently used to sequence both strands of cloned fragment A. Alignment of fragments A and B revealed 100% sequence identity, thereby confirming the location of the translational stop codon and absence of a CTP.

subunit includes a CTP domain. This in turn suggests that the deletion mutation(s) and consequent frameshift that led to expression of a CTP in equids occurred in the equid lineage after divergence of the family Equidae from the family Rhinocerotidae. Thus, it can be postulated from the present studies that if rhino species do express a luteotropin *β* subunit in placenta, it would lack a CTP.

The deduced rnLH/(CG?)*β* aa sequence (see Fig. 3B) suggests a peptide structure typical of mammalian LH*β* subunits (Bousfield et al., 1994). All cysteine residues were found to be in register with the 14 highly conserved cysteines present in the consensus mammalian LH*β* subunit. A single potential consensus glycosylation attachment site is present at Asn^{13}, which is the typical location for oligosaccharide attachment in other mammalian LH*β* subunits (Bousfield et al., 1994). Comparison across the *β* subunit regions (aa +90 to +109) containing the aa sequences thought to be responsible for the unusual dual LH–FSH biological activities of native eLH and eCG (Combarnous, 1992; Moyle et al., 1994) reveals that the corresponding rhino sequence is more similar to the consensus mammalian LH*β* subunit (e.g., pLH*β*) than to the divergent eLH/CG*β* subunit (see Fig. 3B). This outcome was not unexpected based on previous studies demonstrating that the majority of the distinctive aa substitutions between residues +90 and +109 in eLH/CG*β* are also absent in donkey LH and CG (Chopineau et al., 1995; Murphy and Martinuk, 1991), which lack significant dual LH–FSH activity. Hence, the majority of the mutations leading to the unique eLH/CG*β* aa substitutions and function appear to have occurred after the divergence of horses from donkeys, and therefore even longer after the divergence of equids from rhinos.

2.4. Analysis of rnLHβ transcript

In primates and equids, acquisition of the capacity for placental expression of a CG*β* subunit is associated with adoption of an elongated 5′-*UTR* region, compared to the 6–11 bp 5′-*UTR* found in consensus LH*β* transcripts (Sherman et al., 1992; Talmadge et al., 1984). Human CG*β* transcripts bear an approximately 350 nt 5′-*UTR*, whereas in the case of the single *eLH/CGβ* gene, the same elongated 5′-*UTR* (45–62 nt) is present in both placental and pituitary transcripts. While evidence remains circumstantial that adoption of a non-consensus transcriptional initiation site plays a role in conferring the ability of *CGβ* and *LH/CGβ* genes to

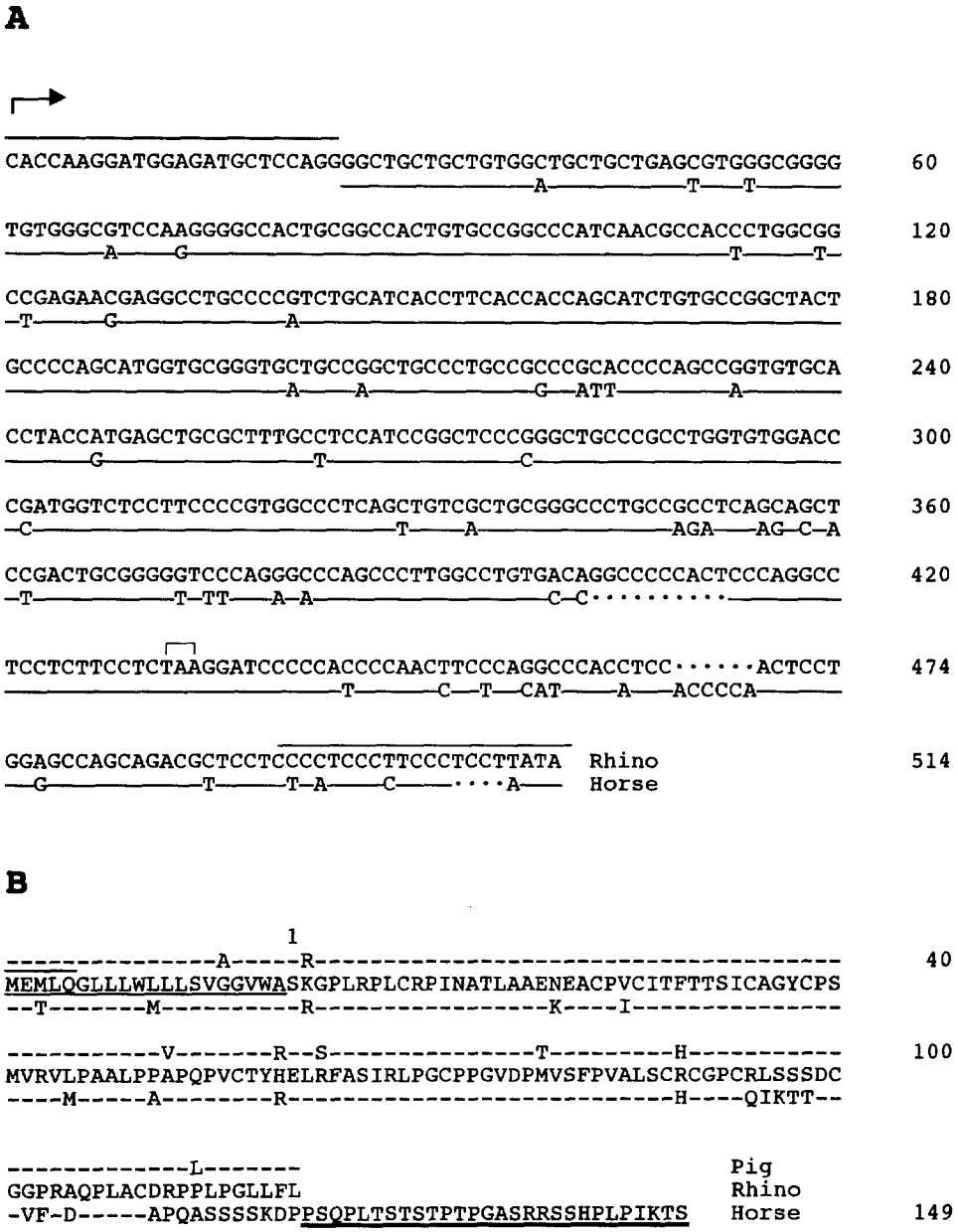

Fig. 3. Comparison of nucleic acid and amino acid sequences of white *rnLHβ* cDNA with porcine and equine sequences. (**A**) Alignment of *rnLHβ* cDNA sequence with that of horse *LH/(CG)β* (Sherman et al., 1992). Only the nt that differ in the horse are shown. Sequences representing amplification primers are overlined. The 5′ primer was porcine based and therefore these nt may not represent exact rhino sequence. The transcriptional start site is indicated by the arrow (see Section 2.4 and Fig. 5). The consensus luteotropin β subunit translational start codon (ATG) begins at nt position 9. The rhino translational stop codon is bracketed and its codon position is consistent with that of the consensus mammalian *LHβ* sequence. This sequence has been submitted to the GenBank database (accession number U72659). (**B**) Deduced aa sequence of white rnLHβ subunit aligned with pLHβ (Ezashi et al., 1990) and eLH/(CG)β. Only aa that differ in the latter two species are shown. The signal peptide sequence is underlined and the aa sequence deduced from porcine 5′ primer is overlined. The equine sequence shows the CTP extension (heavily underlined) that results from frameshift deletion(s) (relative to consensus mammalian sequence) in the region of nt 403–412 (see Fig. 3A).

express in placenta, it was of interest to determine the site of transcriptional initiation in the pituitary for the *rnLH/(CG?)β* gene. To this end, primer extension analysis of LHβ transcripts was performed using as a source of template total RNA prepared from the pituitary glands of one black and one white rhino. Fig. 5 reveals major primer extension products corresponding to an 8 nt 5′-*UTR* for both rhino species. Consistent with the primer extension data are results of the Northern blot analysis (see Fig. 6) which reveal rnLHβ transcripts of approximately the same length as rat LHβ mRNA, the latter bearing a 7 nt 5′-*UTR* (Jameson et al., 1984). Had the rnLHβ pituitary transcript been significantly longer than the consensus LHβ transcript (e.g., rat), the reso-

Table 1
Distance matrix[a], expressed as number of substitutions per 100 bases, for mammalian[b] LHβ and CGβ DNA sequences[c,d]

	hCGβ	hLHβ	dkLH/CGβ	eLH/CGβ	pLHβ	rnLHβ	dLHβ	bLHβ	rLHβ	tLHβ
hCGβ		7	19	21	20	19	22	20	25	45
hLHβ			17	19	17	17	18	18	21	44
dkLH/CGβ				3	10	9	12	12	17	44
eLH/CGβ					12	10	12	13	18	44
pLHβ						8	10	11	14	46
rnLHβ							10	11	15	44
dLHβ								12	15	46
bLHβ									18	46
rLHβ										49
tLHβ										

Ranked distance relative to rhino:
Closest ——— Furthest
Pig--donkey--horse--dog--bovine--rat--human--turkey

[a]Alignments and matrix construction performed with the assistance of Wisconsin Package software, version 8.1, Sept. 1995, Genetics Computer Group, 575 Science Drive, Madison, WI.
[b]h = human, dk = donkey, e = equine, p = porcine, rn = rhinoceros, d = dog, b = bovine, r = rat, t = turkey.
[c]Sequences retrieved from Genbank.
[d]Comparisons included encoding DNA sequence corresponding to codons +1 through +110.

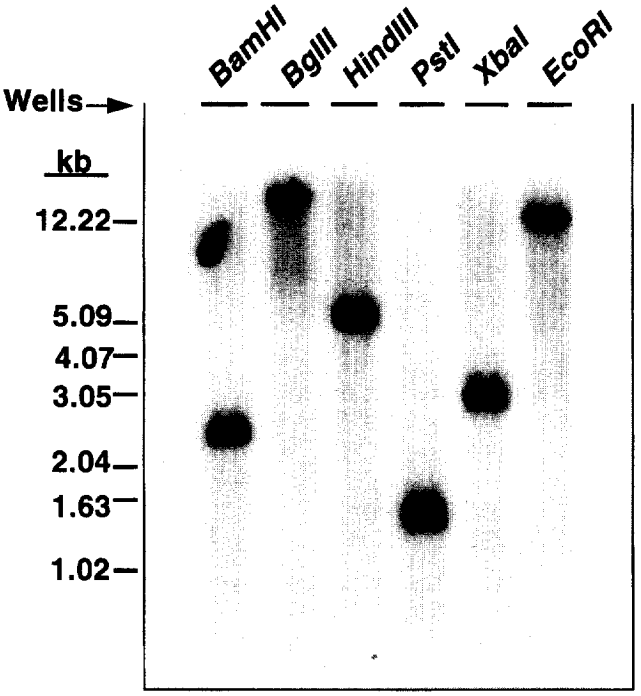

Fig. 4. The simple Southern blot hybridization pattern suggests that Indian rhinoceros (*Rhinoceros unicornis*) has a single *LH/(CG?)β* gene per haploid genome. Genomic DNA was isolated from liver using standard techniques (Strauss, 1995). Restriction enzyme-digested DNA (10 μg) was electrophoresed in 1 × TBE in 0.7% agarose gel at 2.5 V/cm and blotted on Duralon-UV membrane (Stratagene) according to the manufacturer's specifications. The blot was hybridized to ^{32}P-labelled *rnLHβ* cDNA probe (sequence corresponding to codons −20 to +121) prepared by random primed labelling (Multiprime DNA labelling system, Amersham) at 65°C in QuikHyb solution (Stratagene) for 2.5 h. Two washings in 2 × SSC/0.1% SDS at 25°C for 15 min were followed by a final wash in 0.1 × SSC/1% SDS, 60°C for 30 min. The membrane was exposed to autoradiography film (X-omat, Kodak) with an intensifying screen at −70°C for 30 h.

lution potential of the gel/autoradiograph with shorter exposure (data not shown) would have been sufficient to reveal such a difference, assuming conserved 3′-*UTR* and poly-A tail lengths[2]. Taken together, these findings support rejection of the hypothesis that the 5′-*UTR* of rnLHβ pituitary transcripts is elongated compared to the consensus mammalian LHβ transcript. Based on these results, it is proposed that the mutation(s) responsible for the distinctive 5′-*UTR* structure of pituitary *eLHβ* transcripts occurred in the equid lineage after the divergence of the family Equidae from the family Rhinocerotidae.

2.5. Evolutionary and teleological implications

The principal findings of the present study are summarized graphically in the phylogenetic model shown in Fig. 7. Absence of evidence of a mutation leading to CTP expression and rearrangement of the 5′-*UTR* at the *rnLH/(CG?)β* locus supports the contention that these mutations did not occur in perissodactyl evolution until after equids diverged from rhinos. Hence, the data are supportive of a simple evolutionary model in which the *LHβ* gene sequence and locus structures present in the ancestral mammal are generally retained in the ancestral perissodactyl (i.e., prior to radiation of perissodactyl species) and continue into the rhino lineage.

The presence of a CTP and restructured 5′-*UTR* in the only two phylogenetic clads (equids and primates) known to produce CG (Sugino et al., 1987; Murphy and Martinuk, 1991) suggests that mutations leading to these distinctive features confer a selective advantage relative to hormone function and/or the capacity for

[2] A 1 h exposure produced significantly sharper probe-positive bands, making it possible to distinguish transcript length differences of 20–30 bases.

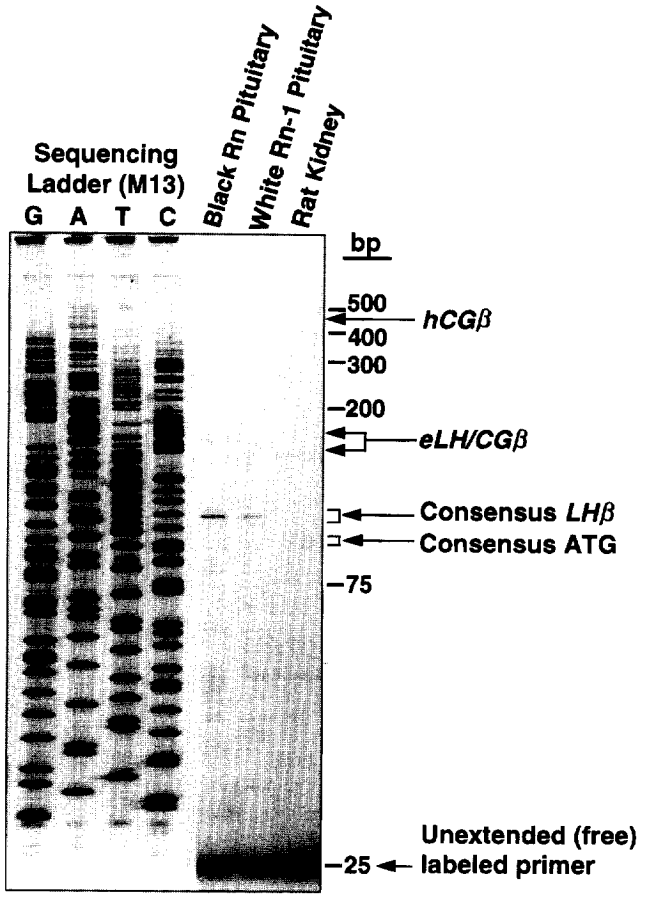

Fig. 5. Primer extension mapping suggests the location of black and white rhinoceros LH/(CG?)β pituitary transcriptional start sites is typical of most other mammalian LHβ transcripts. A 25 nt primer (5′-TGGGCCGGCACAGTGGCCGCAGTGG) corresponding to sequence encoding aa +4 to +12 was end-labelled using T4 polynucleotide kinase according to the manufacturer (Gibco). Total pituitary or control RNA (100 μg) was reverse-transcribed as previously described (Sherman et al., 1992). Terminated primer extension reactions were applied, along with control (M13) sequencing reactions (Sequenase DNA Sequencing Kit; Amersham), to a standard denaturing 8% polyacrylamide sequencing gel (Slatko and Albright, 1995) and electrophoresed for 1.5 h. Following electrophoresis, the gel was fixed, transferred to 3 mm paper, dried and exposed to autoradiography film (X-omat, Kodak) for 55 h. Locations of the consensus translational start codon (ATG), as well as the transcriptional initiation sites for typical mammalian LHβ mRNAs, eLH/CGβ mRNA and hCGβ mRNA are indicated. The distance (number of nt) from the 3′ end of the rhino extension primer to the 3′ end of the rhino extension products (i.e., transcriptional start site) was determined by subtracting the length of the primer (25 nt) from the total length of the extension products (102 nt). The precise length of the rhino extension products was determined by direct comparison with the adjacent sequencing ladder standard (M13). Black Rn, 20-year-old, female, black rhino (*Diceros bicornis*); white Rn-1, 31-year-old, female, southern white rhino (*C. s. simum*; same animal as in Fig. 2).

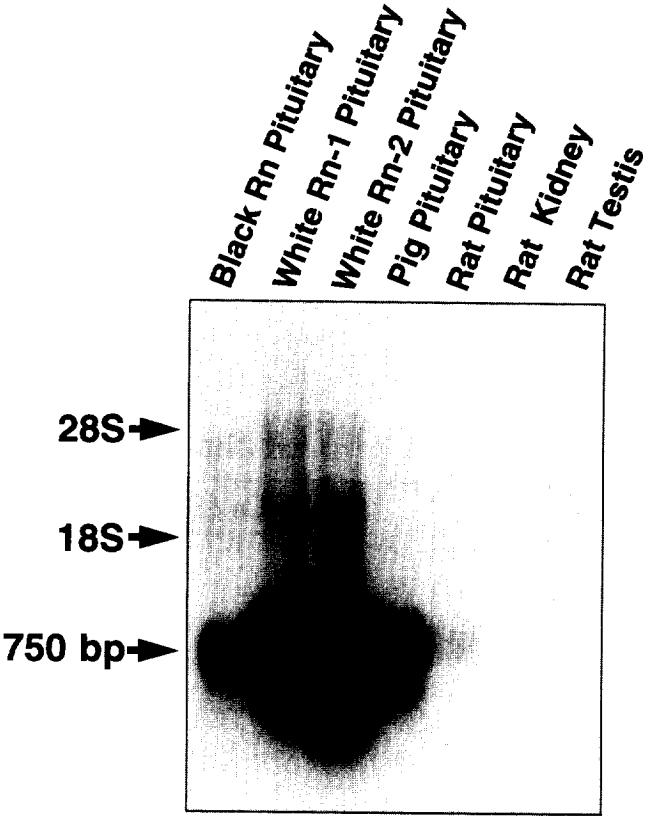

Fig. 6. Northern blot analysis demonstrates that black and white rhinoceros pituitary LHβ transcript lengths (approximately 750 nt) are similar to other species. A guanidinium method (Kingston et al., 1995) for total RNA isolation was used to prepare pituitary RNA and negative control RNA. Samples (10 μg) were electrophoresed in a 2.2 M formaldehyde/1.5% agarose gel for 14 h at 1.5 V/cm (Brown, 1995). Blotting, probe hybridization and film exposure were performed as described in Fig. 4 with the exception of an 11 h exposure of membrane to film. Black Rn and white Rn-1 as in Fig. 5; white Rn-2, 5-year-old, male, southern white rhino (*C. s. simum*).

placental expression. However, it remains possible that a luteotropin β gene lacking these two attributes could be placentally expressed in extinct or yet to be studied extant species (such as rhinos), since obligatory roles for a CTP and altered proximal 5′-flanking sequence arrangements in CG function and expression have yet to be demonstrated. Indeed, according to one plausible scenario of perissodactyl evolution, a CGβ subunit lacking a CTP could have evolved if (1) 5′-flanking/promoter mutation(s) (other than 5′-*UTR* elongation) were required for activating placental *LH/CG*β gene transcription, and (2) these promoter mutation(s) preceded the deletion mutation leading to CTP expression. If such were the case, it follows that rhinos (or some other perissodactyl) could have diverged from equids *between* the occurrences of these two mutational events, thereby effecting placental expression of a CTP-less rhino CGβ subunit. The alternative scenario is that rhinos diverged prior to both mutational events, regardless of the order of the mutations. This would result in retention of a consensus, CTP-less, mammalian LHβ gene that is expressed only in pituitary (i.e., the ancestral condition). While each of the above evolutionary pathways is consistent with our findings, the present study

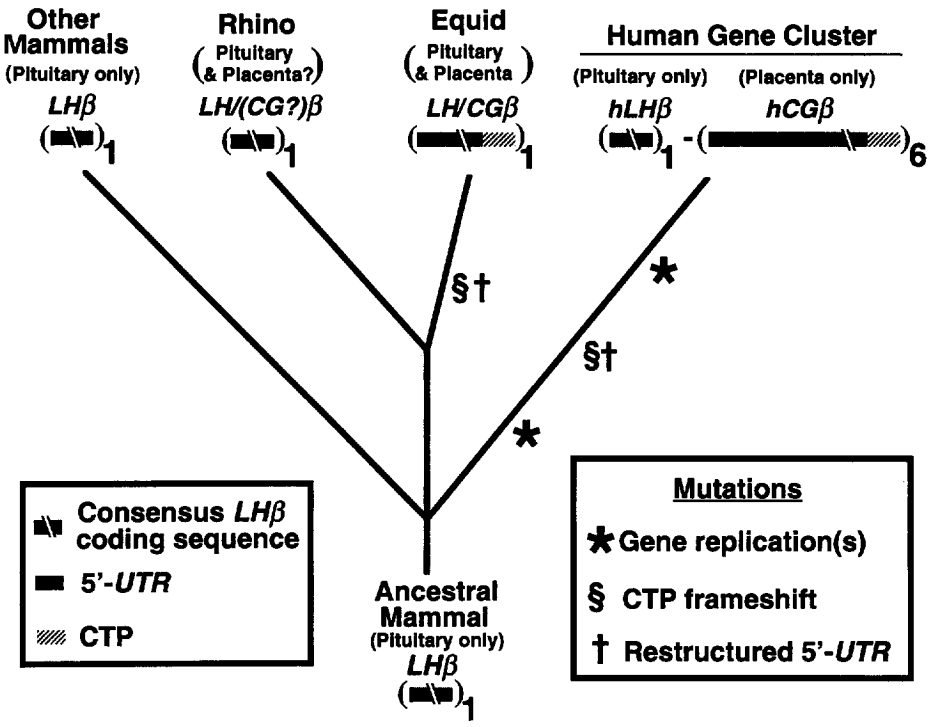

Fig. 7. Proposed evolutionary model for the rhinoceros luteotropin β subunit gene locus.

does indicate the improbability of all scenarios in which the divergence of rhinos occurred after CTP-inducing mutation(s). In this case, expression of a CTP domain, or at least vestiges of a nt rearrangement, would be evident in both rhino and equid lineages, and such is not the case. Indeed, there is almost complete conservation of rhino and pig aa sequence (see Fig. 3B) in the region where CTP-inducing deletion mutation(s) occurred in the horse.

Finally, consideration must also be given to the possibility that the capacity to express placentally the *LH/(CG?)β* gene evolved autonomously in rhinos following their divergence from equids. We have previously reported evidence supporting the contention that CGβ subunit expression evolved independently in equids and primates, an example of convergent evolution (Sherman et al., 1992). Moreover, there are data suggesting that guinea-pigs may express placental CG (Bambra et al., 1984). If ultimately verified, the existence of guinea-pig CG would represent another example of convergent evolution at the luteotropin β gene locus. Thus, while it would seem an unlikely coincidence, the possibility that placental CGβ expression evolved independently in different branches of the perissodactyl lineage cannot be ruled out. It is anticipated that further comparative analyses of luteotropin β gene promoter sequences and expression patterns will facilitate elucidation of the principal molecular mechanisms responsible for transcriptional activation of LHβ, CGβ and LH/CGβ genes in pituitary and placental tissues.

3. Conclusions

(1) The *rnLH/(CG?)β* locus was determined to contain a single-copy gene that encodes a consensus-like mammalian LHβ peptide and generates a pituitary transcript with an 8 bp *5'-UTR*.

(2) Rhino *LH/(CG?)β* cDNA sequence, transcript and gene locus structures are typical of the consensus mammalian *LHβ* condition. This suggests that evolutionary divergence of the family Rhinocerotidae from the family Equidae occurred prior to the mutational events responsible for *5'-UTR* rearrangement and acquisition of CTP expression in the equid lineage.

(3) While the findings of the present study imply that rhino luteotropin β gene expression may be limited to the pituitary gland, the possibility cannot be discounted that the promoter of the *rnLH/(CG?)β* gene is active in rhino placenta, producing a CTP-less CGβ subunit.

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