Purification and Partial Characterization of the Cyanogen Bromide Fragments of Ovine Placental Lactogen†

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ABSTRACT: Ovine placental lactogen (oPL) shares a number of properties with both ovine prolactin (oPRL) and ovine growth hormone (oGH). To further delineate the structural relationships among these hormones, oPL was subjected to specific cleavage at methionyl residues with cyanogen bromide (CNBr) to obtain a series of smaller fragments for chemical studies. Amino acid analysis of the unseparated fragments indicated a 92% conversion of methionine to homoserine and homoserine lactone. The six unique CNBr fragments produced by cleavage at five methionyl residues were purified by gel chromatography in 20% acetic acid. The combined composition of the purified fragments, based on 24-h hydrolyses, equals the composition of oPL except for reduced recovery of valine and isoleucine. The COOH-terminal fragment of oPL, identified by its lack of methionine, contains three half-cystine residues, one of which, prior to oxidation, is joined by a disulfide bond to another CNBr peptide to form a large disulfide loop in the intact protein. The amino-terminal peptide, identified on the basis of amino terminal sequence data identical with that observed with intact oPL, contains two half-cystine residues; results of Edman degradation suggest that they are located at positions very similar to the first two half-cystine residues of oPRL. Placement of the other CNBr fragments was based on compositional similarities with peptides of oPRL. These results support the hypothesis that oPL is structurally similar to oPRL as well as oGH and suggest that the subprimate placental lactogens originated from the prolactin gene rather than from the growth hormone gene as postulated for primates.

We have found that ovine placental lactogen (oPL;[1] see Hurley et al., 1977) shares important physical and chemical features with ovine prolactin (oPRL) as well as with ovine growth hormone (oGH). These similarities are consistent with reports of ours (Handwerger et al., 1974) and of Chan et al. (1976) which showed that oPL has biological potencies comparable to those of both oPRL and oGH in vivo and in vitro. However, these studies suggest a closer structural resemblance of oPL to oPRL than was expected from known relationships among the corresponding primate hormones. The placental lactogen of humans (hPL) and of monkeys (mPL) both closely resemble primate growth hormones, rather than primate prolactins (Bewley and Li, 1974). The extensive similarities of primary sequence have led to the proposal (Dayhoff et al., 1975) that hPL originated from a duplication of the growth hormone gene well after the differentiation of the principal mammalian orders (Wallis and Davies, 1976).

We report here the results of studies on specific cleavage of oPL at methionyl residues which confirm earlier evidence of a close structural relationship between oPL and oPRL. This study suggests that the subprimate placental lactogens, unlike those of the primates, evolved from the prolactin line of the growth hormone–prolactin family.

Materials and Methods

Ovine placental lactogen (oPL) was purified as described in the preceding paper (Hurley et al., 1977). Sephadex G-50 (Superfine) and G-100 (superfine) were purchased from Pharmacia. Cyanogen bromide (CNBr; lot no. 08243-7), β-mercaptoethanol, and phenylthiohydantoin (Pth) amino acid standards were Sequanal grade from Pierce Chemical Co. Iodo[1-^{14}C]acetic acid (lot no. 861-029; specific activity: 13.3 Ci/M) was from New England Nuclear. Hydrolyses were carried out at reduced pressure for 24 h in 6 N HCl (Ultrex, J. T. Baker Co.) at 110 °C. Amino acid analyses were performed on a Beckman 121M microcolumn amino acid analyzer using buffers and ninhydrin purchased from Beckman. Automated sequencing procedures and identification and quantitation of Pth-amino acids were performed as described in the accompanying paper (Hurley et al., 1977).

Purified oPL was dissolved in 70% formic acid at a concentration of 10–12 mg/mL and a twofold excess by weight of solid CNBr was added (Gross, 1967). After 24 h with stirring at room temperature, the solution was diluted tenfold with water and lyophilized. The dried powder was twice resuspended in water and lyophilized to remove volatile reaction products.

The products of CNBr cleavage were separated on a column, 2.5 × 95 cm, of Sephadex G-50 (superfine) equilibrated and eluted at 4 °C with 20% acetic acid. Further purification of individual fragments was obtained by chromatography on columns of Sephadex G-50 (superfine) or G-100 (superfine) as described in figure legends. Samples were applied to Sephadex columns in 60% acetic acid.

Reduction and alkylation of disulfide bonds was carried out by a modification of the method of Hirs (1967a). Peptide (15 nmol) was reduced at 50 °C in the sequencer cup with 1500 nmol (50-fold molar excess over thiol groups) of β-mercapto-

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[1] Abbreviations used: oPL, ovine placental lactogen; oPRL, ovine prolactin; oGH, ovine growth hormone; CNBr, cyanogen bromide; Pth, phenylthiohydantoin; CM, carboxymethyl; GC, gas chromatography; TLC, thin-layer chromatography.

TABLE I: The Amino Acid Compositions of the Cyanogen Bromide Fragments of oPL.[a]

Amino acid	Cyanogen bromide fragments 1C	3	5	6	7	Total	fragments	Unseparated oPL
Lys	5.0(5)	3.0(3)	1.9(2)	0.8(1)	3.1(3)	14	14.2(14)	14
His	2.2(2)		1.2(1)	0.8(1)		4	4.0(4)	4
Arg	2.1(2)	3.7(4)	0.7(1)	1.7(2)	0.9(1)	10	9.7(10)	10
Asp	7.9(8)	7.3(7)	0.7(1)	1.3(1)	2.4(2)	19	18.6(19)	19
Thr	2.6(3)	2.7(3)	0.8(1)	1.7(2)	0.9(1)	10	9.5(10)	10
Ser	7.2(7)	3.9(4)	0.8(1)	1.9(2)	1.0(1)	15	15.1(15)	15
Glu	8.7(9)	3.7(4)		4.6(5)	5.7(6)	24	23.7(24)	24
Pro	3.1(3)	1.0(1)	1.0(1)	2.9(3)	2.0(2)	10	10.2(10)	10
Gly	4.2(4)	3.4(3)	3.9(4)	2.1(2)	2.1(2)	15	15.3(15)	15
Ala	6.4(6)	2.1(2)	1.1(1)	1.8(2)	2.2(2)	13	12.9(13)	13
½-cystine[b]	3.8(4)				1.7(2)	6	4.7(5)[c]	6
Val	1.8(2)		0.6(1)	1.3(1)	1.6(2)	6	7.3(7)	12
Met[d]	1.3(1)	0.6(1)	1.1(1)	1.0(1)	1.2(1)	5	0.4[e]	5
Ile	0.8(1)		0.7(2)[f]	0.6(1)		4	6.1(6)	10
Leu	4.6(5)	3.7(4)	0.8(1)	2.1(2)	0.7(1)	13	12.6(13)	13
Tyr	0.9(1)	1.4(1)	1.0(1)	1.0(1)		4	3.8(4)	4
Phe	1.8(2)	2.1(2)		1.6(2)	0.6(1)	7	7.1(7)	7
Trp		0.7(1)	0.6(1)			2	nd[g]	2
Final yield (mg)	3.4	3.1	3.2	2.1	1.2		21.2	

[a] Values for each amino acid are based on 24-h hydrolyses with assumed integral numbers in parentheses. [b] Determined as cysteic acid. [c] Determined as half-cystine. [d] Determined as the sum of homoserine and homoserine lactone. [e] Determined as methionine. [f] Sequence data on fragment 3 (Table IV) shows two isoleucine residues. [g] nd, not determined.

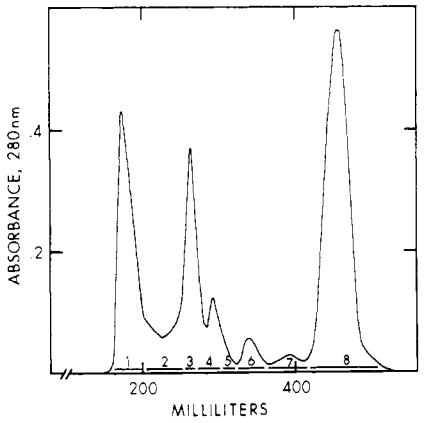

FIGURE 1: The initial separation of the products of cleavage of oPL with cyanogen bromide. Unseparated fragments were dissolved in 4.0 mL of 60% acetic acid and applied to a column, 2.5 × 90 cm, of Sephadex G-50 (superfine) equilibrated and eluted at 4 °C with 20% acetic acid at 15 mL/h. Eluted material was pooled as indicated by the numbered bars for further purification.

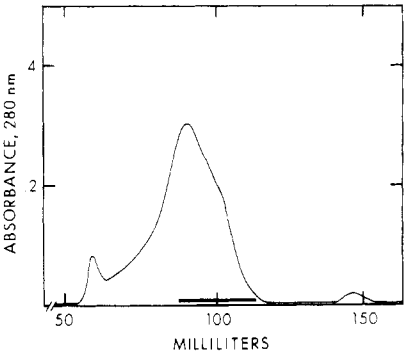

FIGURE 2: Rechromatography of cyanogen bromide pool 1. Pool 1 (Figure 1) was rechromatographed on a column, 1.5 × 82 cm, of Sephadex G-150 equilibrated and eluted with 20% acetic acid. The solid bar, fraction 1C, represents material pooled for further characterization.

ethanol for 30 min in 0.1 M Quadrol buffer. Unlabeled iodoacetic acid and iodo[1-^{14}C]acetic acid (4:1; 1400 nmol total) were added in water and the reaction was carried out at 50 °C for 10 min in the dark. Excess reagents were removed by vacuum and extraction with benzene, ethyl acetate, and butyl chloride.

Results

Amino acid analysis (Table I) showed that 92% of the methionyl residues had reacted with CNBr as indicated by conversion to homoserine and homoserine lactone. The reaction products were separated initially on a column of Sephadex G-50 (superfine) to yield six peaks which were pooled as indicated in Figure 1.

Pool 1 was rechromatographed on a column of Sephadex G-150 to separate aggregated and partially cleaved material from authentic methionyl peptides (Figure 2). Aggregated material was eluted at the void volume, followed by smaller material preceding the principal peak (K_{av} = 0.38). Amino acid analysis of the material in pool 1C (Table I) shows the presence of 1 methionine (as homoserine and homoserine lactone) and 4 half-cystine residues. Since a peptide of similar composition was observed in structural studies of bGH (Fellows and Rogol, 1969) and bPR (Fellows et al., 1970), it was thought that fragment 1C contained two CNBr fragments linked by a disulfide bond, one of which, lacking a methionine residue, represented the carboxyl terminus of the intact protein. Therefore, fragment 1C was oxidized with performic acid (Hirs, 1967b) and submitted to rechromatography on a column of Sephadex G-100 (superfine) (Figure 3).

The amino acid composition (Table II) of the first peak eluted, pool 1C1, is identical with that of pool 1C (Figure 2; Table I), indicating that it is unoxidized material from the parent pool. The composition of pool 1C2 (Table II) includes one methionine and one cysteic acid residue, while fragment 1C3 contains 3 cysteic acid residues but no methionine. As shown in Table II, the sum of the amino acids contained in

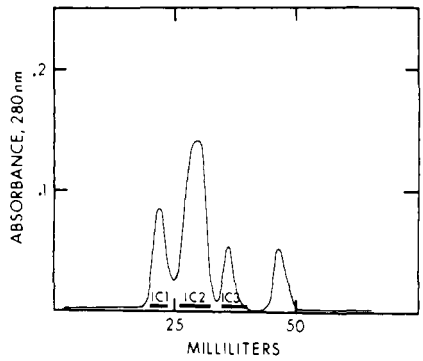

FIGURE 3: Chromatography of cyanogen bromide fragment 1C after oxidation with performic acid. Cyanogen bromide fragment 1C (Figure 2) after oxidation with performic acid was dissolved in 1.0 mL of 60% acetic acid and applied to a column, 0.9 × 81 cm, of Sephadex G-100 (superfine) equilibrated and eluted at 4 °C with 20% acetic acid at 1.0 mL/h. Eluted material was pooled as indicated for further characterization.

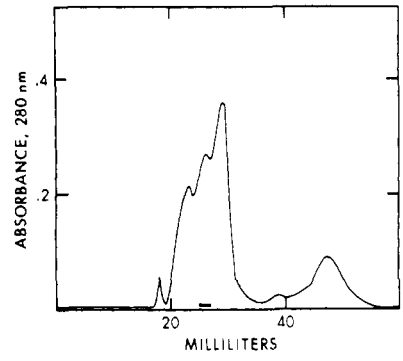

FIGURE 4: Rechromatography of pool 2 on Sephadex G-50. Material from pool 2 (Figure 1) was rechromatographed at 4 °C on a column, 0.9 × 80 cm, of Sephadex G-50 (superfine) equilibrated and eluted with 20% acetic acid at 1.5 mL/h. Material indicated by the bar (pool 2A) was hydrolyzed for amino acid analysis (Table III).

TABLE II: The Amino Acid Compositions of Cyanogen Bromide Fragments 1C1, 1C2, and 1C3.[a]

Amino acid	Cyanogen bromide fragments 1C1	1C2	1C3
Lys	5.1(5)	2.8(3)	1.9(2)
His	2.2(2)	0.8(1)	0.9(1)
Arg	1.8(2)	2.0(2)	
Asp	8.0(8)	6.2(6)	2.1(2)
Thr	2.7(3)	2.2(2)	1.1(1)
Ser	6.9(7)	5.2(5)	1.8(2)
Glu	9.2(9)	5.9(6)	2.9(3)
Pro	3.2(3)	3.3(3)	
Gly	3.7(4)	3.2(3)	1.1(1)
Ala	6.1(6)	3.3(3)	3.2(3)
½-cystine	3.4(3)[b]	0.7(1)[c]	2.8(3)[c]
Val	2.3(2)	2.1(2)	
Met[d]	0.8(1)	1.1(1)	
Ile	1.1(1)	1.2(1)	
Leu	4.8(5)	4.9(5)	
Tyr	1.0(1)	0.9(1)	
Phe	2.3(2)	1.7(2)	
Trp			

[a] Values for each amino acid are based on 24-h hydrolyses with assumed integral numbers in parentheses. [b] Determined as half-cystine. [c] Determined as cysteic acid. [d] Determined as the sum of homoserine and homoserine lactone.

TABLE III: The Amino Acid Compositions of Cyanogen Bromide Fragments 1C2, 3, and 7 Compared with That of Fraction 2A.[a]

Amino acid	Cyanogen bromide fragments 1C2	3	7	Total	Fraction 2A
Lys	3	3	3	9	8.9(9)
His	1	1		2	1.3(2)
Arg	2	4	1	7	7.0(7)
Asp	6	7	2	15	11.5(12)
Thr	2	3	1	6	5.9(6)
Ser	5	4	1	10	10.0(10)
Glu	6	4	6	16	18.0(18)
Pro	3	1	2	6	7.1(7)
Gly	3	3	2	8	11.1(11)
Ala	3	2	2	7	10.0(10)
½-cystine	1		2	3	1.7(2)
Val	2	1	2	5	6.9(7)
Met	1	1	1	3	1.5(2)
Hse (lactone)					0.7(1)
Ile	1	2		3	3.4(3)
Leu	5	4	1	10	11.5(11)
Tyr	1	1		2	4.9(5)
Phe	2	2	1	5	5.0(5)
Trp		1		1	nd[b]

[a] Values are expressed as residues per molecule with assumed integral numbers in parentheses. [b] nd, not determined.

TABLE IV: The Amino-Terminal Sequence of Cyanogen Bromide Fragment 3.[a]

Cycle No.	Pth-amino acids detected by GC	TLC
1	Ile (63 nm)	Ile
2	Ala (62 nm) Ile (7 nm)	Ala
3		Arg
4	Gly (31 nm)	Gly
5		
6	Tyr (37 nm)	Tyr
7	Asp (7 nm) Tyr (8 nm)	Asn
8	Ile (32 nm)	Ile
9	Lys (27 nm)	Lys

[a] Fragment 3, 71 nmol, was submitted to automated Edman degradation.

fragments 1C2 and 1C3 is identical with that of fragment 1C (Table I).

Pool 2 (Figure 1) was rechromatographed on a column of Sephadex G-50 (superfine) equilibrated and eluted with 20% acetic acid. The elution pattern (Figure 4) consists principally of material from peaks 1 (K_{av} = 0.20) and 3 (K_{av} = 0.37, Figure 1) but also contains a third component migrating between the two which is designated 2A (K_{av} = 0.28). The amino acid analysis of fragment 2A (Table III) shows the presence of unaltered methionine as well as homoserine and its lactone. The combined compositions of three other CNBr peptides approximate that of fragment 2A (Table III). Thus, fragment 2A is not considered to be a unique methionyl peptide of oPL but apparently arose from incomplete methionyl cleavage and nonspecific cleavage of a disulfide bond. A similar fragment has been observed in studies of the cyanogen bromide peptides of bGH (Fellows and Rogol, 1969).

Pool 3 (Figure 1) was rechromatographed on a column, 0.9 × 80 cm, of G-50 (superfine) equilibrated and eluted with 20% acetic acid to yield a single symmetrical peak. Its amino acid composition is shown in Table I. The results of sequence analyses are presented in Table IV. No Pth-amino acids were

detected by thin-layer or gas chromatography at position 5, which may be occupied by a labile residue destroyed during conversion and analysis (Niall, 1973).

Pools 4, 5, and 6. Pool 4 (Figure 1) was rechromatographed on a column, 0.9 × 80 cm, of Sephadex G-50 (superfine) and revealed no constituents other than material corresponding to fractions 3 (K_{av} = 0.31) and 5 (K_{av} = 0.41) of Figure 1. Fraction 5 (Figure 1) was rechromatographed on a column, 0.9 × 75 cm, of Sephadex G-50 (superfine) equilibrated and eluted with 20% acetic acid to yield a major peak which was pooled to exclude a small shoulder on the leading edge. The amino acid composition of CNBr fragment 5 is shown in Table I. Pool 6 (Figure 1), characterized without further purification, had the amino acid composition shown in Table I. It was submitted to Edman degradation, yielding the results given in Table V.

Pool 7. Fragment 7 (15 mm), containing two half-cystines (Table I), was submitted to Edman degradation after reduction with β-mercaptoethanol and alkylation with [1-^{14}C]iodoacetic acid. Cycle 1 yielded 9 nmol of Pth-valine by gas–liquid chromatography and both valine and Pth-alanine on thin-layer chromatography. At cycle 2, Pth-phenylalanine (8 nmol) was observed by both chromatographic methods. These data are consistent with the data obtained by Edman degradation of intact, performic acid oxidized oPL (see accompanying paper: Hurley et al., 1977).

The cysteic acid content of fragment 7 suggested that a cystine residue was present near the amino terminus of oPL. In an attempt to locate its position, aliquots of the ethyl acetate phase (containing Pth-S-[^{14}C]carboxymethylcysteine) were counted with a Nuclear-Chicago liquid scintillation spectrometer with a 90% efficiency for ^{14}C. A sharp peak of radioactivity was observed at cycle 10, although the peak expected at cycle 3 from degradation of intact performic acid oxidized oPL (see Hurley et al., 1977) was not distinguished from a high background.

Pool 8 (Figure 1), containing no amino acids above background levels, consists of nonvolatile reaction products.

Amino acid composition of the CNBr fragments of oPL (Table I) accounts for the entire composition of the protein with two exceptions: the sums of both valine and isoleucine are each short of the expected total, probably the results of incomplete hydrolysis, due to steric hindrance of their branched side chains (Inouye et al., 1970).

Discussion

Since, except for two amino acids, the compositions of the fragments given in Table I account for all of the oPL molecule, it is possible to propose an alignment of the fragments as they occur in the intact protein. Fragment 1C3 (Figure 3, Table II), the only fragment without methionine, is identified as the carboxyl terminus of the protein. Because of the physical association of fragments 1C3 and 1C2 prior to reduction of the disulfide bonds, the intact protein is presumed to contain a disulfide loop linking the carboxyl terminal peptide, 1C3, with an internal methionyl peptide. 1C2. Moreover, fragment 1C3 contains two additional half-cystine residues, one of which, as indicated by carboxypeptidase digestion (see Hurley et al., 1977), is located near the COOH terminus of the protein, forming a second disulfide loop with the third half-cystine residue of fragment 1C3. Taken together, these data suggest that oPL has a small disulfide loop near the carboxyl terminus and a second disulfide bond connecting the carboxyl terminus fragment 1C3, with a larger internal fragment, 1C2.

The amino acid composition of oPL indicates that two more half-cystine residues are present and they are found in a single peptide, cnbr fragment 7. Since fragment 7 shows the same amino-terminal heterogeneity seen in the intact protein, it is assigned to the amino terminus of oPL. The cystine content of fragment 7 and its small size (27 residues) indicates that a disulfide loop is present near the amino terminus of intact oPL but exact location of the residues is complicated by the heterogeneity of both the fragment and the intact protein. When intact oPL was sequenced after oxidation with performic acid (accompanying paper), Pth-cysteic acid was prominent at cycle 3. In an attempt to identify the location of the second half-cystine, the amino-terminal methionyl peptide (CNBr fragment 7) was reduced and alkylated with [1-^{14}C]iodoacetic acid. The results of analysis for radiolabeled cleavage products, while not definitive, indicate the presence of a [1-^{14}C]carboxymethylcysteine residue at position 10.

TABLE V: The Amino-Terminal Sequence of Cyanogen Bromide Fragment 6.[a]

Cycle No.	Pth-amino acids detected by GC	Pth-amino acids detected by TLC
1	Gly (41 nm)	Gly
2		Thr
3	Tyr (107 nm)	Tyr
4	Gly (53 nm)	Gly
5	Pro (58 nm)	Pro
6		Arg
7	Ala (27 nm)	Ala
8	Glu (12 nm)	Gln

[a] Fragment 6, 98 nm, was submitted to automated Edman degradation.

While the positions of the half-cystine residues cannot be assigned unequivocally, the evidence discussed above suggests that they occupy positions 3 and 10 from the amino terminus. This is consistent with the amino terminal sequence of oPRL, in which a disulfide bond joins residues 4 and 11. In addition, the size of the amino terminal methionyl peptide in oPL, 27 residues, is similar to that of the corresponding peptide in oPRL, 24 residues, and much larger than that of oGH, 5 residues.

Cyanogen bromide fragments 3, 6, and 5 are placed within the intact protein in that order. The amino acid composition of the partially purified fragment 2A (Figure 4, Table II) is sufficiently similar to the combined compositions of the unique fragments 7, 1C2 and 3, to suggest that they are joined together in the native protein. Moreover, placing fragment 3 within the large disulfide loop adjacent to fragment 1C2 provides homology between the first tryptophan residue of oPL and the tryptophan residues of oGH and oPRL at positions 86 and 90, respectively. The other two fragments, 6 and 5A, can be arranged to complete the large disulfide loop and provide homology between the tryptophan of fragment 5A and the second tryptophan of oPRL at position 149. This is shown schematically and compared with oGH and oPRL in Figure 5.

The structural properties of oPL indicate that this hormone bears important similarities to both growth hormone and prolactin. It is similar or identical with oGH in isoelectric point, carboxyl-terminal sequence, amino-terminal heterogeneity, and some antigenic determinants and biological properties (Handwerger et al., 1974; Chan et al., 1976). On the other hand, it clearly resembles prolactin with regard to content of half-cystine and tryptophan, its lactogenic and mammotropic activities (Handwerger et al., 1974), and the presence of a disulfide loop near the amino terminus.

Thus, while the primate placental lactogens so far charac-

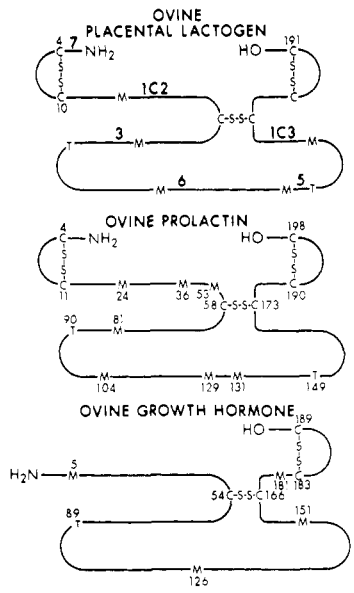

FIGURE 5: Schematic representation of the structures of oPL, oPRL, and oGH. The structure proposed for oPL is based on studies of the intact hormone and of its cyanogen bromide fragments. (C-S-S-C) Disulfide bonds joining cysteine residues; (M) methionyl residues; (T) tryptophanyl residues. The bold letters and numbers of oPL structure denote the cyanogen bromide fragments. Other numbers denote residue portions from the NH_2 terminus.

terized are structurally similar to the primate growth hormones, the placental lactogen from sheep bears important structural similarities to oPRL as well. oPL does not appear unique in this respect. Bolander and Fellows (1976a) have found that the placental lactogen from cattle is structurally intermediate between bovine prolactin and growth hormone and, like oPL, contains 6 half-cystine and 2 tryptophan residues. They have also shown that the amino acid composition of rabbit placental lactogen is similar to that of rabbit prolactin including 6 half-cystine and 2 tryptophan residues (Bolander and Fellows, 1976b). Taken together, these reports suggest that, among the subprimates, the placental lactogens have arisen through duplication of the prolactin gene, perhaps very early in the evolution of eutherian mammals.

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